



University of Kentucky
UKnowledge

University of Kentucky Doctoral Dissertations

Graduate School

2005

EFFECTS OF GLIAL CELL LINE-DERIVED NEUROTROPHIC FACTOR (GDNF) ON STEM/PROGENITOR CELL PROLIFERATION AND DIFFERENTIATION

Yan Chen

University of Kentucky, ychen7@uky.edu

[Right click to open a feedback form in a new tab to let us know how this document benefits you.](#)

Recommended Citation

Chen, Yan, "EFFECTS OF GLIAL CELL LINE-DERIVED NEUROTROPHIC FACTOR (GDNF) ON STEM/PROGENITOR CELL PROLIFERATION AND DIFFERENTIATION" (2005). *University of Kentucky Doctoral Dissertations*. 233.

https://uknowledge.uky.edu/gradschool_diss/233

This Dissertation is brought to you for free and open access by the Graduate School at UKnowledge. It has been accepted for inclusion in University of Kentucky Doctoral Dissertations by an authorized administrator of UKnowledge. For more information, please contact UKnowledge@lsv.uky.edu.

ABSTRACT OF DISSERTATION

Yan Chen

The Graduate School
University of Kentucky
2005

EFFECTS OF GLIAL CELL LINE-DERIVED NEUROTROPHIC FACTOR
(GDNF) ON STEM/PROGENITOR CELL
PROLIFERATION AND DIFFERENTIATION

ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Medicine
at the University of Kentucky

By
Yan Chen
Lexington, Kentucky

Director: Dr. Don Gash, Professor of Anatomy and Neurobiology

Lexington, Kentucky
2005

Copyright © Yan Chen 2005

ABSTRACT OF DISSERTATION

EFFECTS OF GLIAL CELL LINE-DERIVED NEUROTROPHIC FACTOR (GDNF) ON STEM/PROGENITOR CELL PROLIFERATION AND DIFFERENTIATION

Stem/progenitor cells are present in the adult brain; they undergo constant proliferation and differentiate into mature neurons in certain brain areas, a phenomenon called neurogenesis. This study investigated the effects of GDNF, a potent trophic factor of dopaminergic neurons, on neurogenesis in the brain. Nestin and 5-Bromo-2'-deoxyuridine (BrdU) were used as stem/progenitor cells markers.

First, we observed extensive bilateral increases of stem/progenitor cells in the dentate gyrus and substantia nigra after continuous infusion of GDNF into the normal rat brain. However, none of the BrdU+ cells showed neuronal features in the substantia nigra as characterized by immunocytochemical procedures. Next, we identified the morphology of BrdU+ cells after infusing the marker into the brain. While the procedures increased the BrdU labeling, neurogenesis was not observed in the basal ganglia. Under electron microscope, the BrdU+ cells either were undifferentiated or showed characteristics of astrocytes. This observation is consistent with suggestions that astrocytes serve as multipotent progenitors. Later, we repeated GDNF intrastriatal infusion one month after a severe 6-hydroxydopamine (6-OHDA) lesion. The number of

BrdU+ cells was significantly higher in the GDNF recipients in the ipsilateral substantia nigra and both sides of the dentate gyrus. However, no neurogenesis was observed. In addition, motor functions were not improved by GDNF treatment. Thus, we measured the effects of GDNF administration directly into the substantia nigra six hours before a partial 6-OHDA lesion. HPLC measurements of dopamine and its metabolites showed a significant increase of tissue level in the substantia nigra and striatum, respectively. Despite this, no newly generated dopaminergic neurons was detected in the basal ganglia.

Taken together, our studies investigated the effects of GDNF on adult stem/progenitor cells in normal and lesioned rat brain. For the first time, we demonstrated that GDNF promoted their proliferation in the dentate gyrus, suggesting it has a role in neurogenesis and the function of learning and memory. In each scenario, GDNF promoted stem/progenitor cell proliferation, but failed to induce neurogenesis in the substantia nigra. We believed that the local microenvironment in the substantia nigra may prevent the stem/progenitor cells to mature into functional neurons.

KEYWORDS: GDNF, Stem Cell, Progenitor Cell, Dentate Gyrus, Substantia Nigra.

Yan Chen

March 7, 2005

EFFECTS OF GLIAL CELL LINE-DERIVED NEUROTROPHIC FACTOR
(GDNF) ON STEM/PROGENITOR CELL
PROLIFERATION AND DIFFERENTIATION

By

Yan Chen

Dr. Don M. Gash

Director of Dissertation

Dr. Douglas J. Gould

Director of Graduate Studies

March 7, 2005

RULES FOR THE USE OF DISSERTATIONS

Unpublished dissertations submitted for the Doctor's degree and deposited in the University of Kentucky Library are as a rule open for inspection, but are to be used only with due regard to the rights of the authors. Bibliographical references may be noted, but quotations or summaries of parts may be published only with the permission of the author, and with the usual scholarly acknowledgements.

Extensive copying or publication of the dissertation in whole or in part requires also the consent of the Dean of the Graduate School of the University of Kentucky.

A library that borrows this dissertation for use by its patrons is expected to secure the signature of each user.

NAME

DATE

DISSERTATION

Yan Chen

The Graduate School
University of Kentucky

2005

EFFECTS OF GLIAL CELL LINE-DERIVED NEUROTROPHIC FACTOR
(GDNF) ON STEM/PROGENITOR CELL
PROLIFERATION AND DIFFERENTIATION

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Medicine
at the University of Kentucky

By
Yan Chen

Lexington, Kentucky

Director: Dr. Don Gash, Professor of Anatomy and Neurobiology

Lexington, Kentucky

2005

Copyright © Yan Chen 2005

Dedicated to my parents

Dr. Kequan Chen

Prof. Zhongrong Lu

Acknowledgements

I would like to acknowledge many people toward the completion of my graduate study. Foremost, thanks go to my mentor, Dr. Don Gash, for his invaluable support, guidance and encouragement during my school years. His understanding and patient counseling have been a key force that helped me get through many difficulties and frustrations. I am very grateful to have him as my mentor; they have taught me not only to be a good scientist, but also to be a strong person in life.

My sincere thanks also go to Dr. James Geddes, Dr. Wayne Cass, Dr. Anders Andersen, and Dr. Susan Barron for serving as my committee members and to Dr. Michael Bardo for serving as the outside examiner for my dissertation. Their valuable input and scientific guidance have been critical for my research.

I would also like to thank people in the lab: Dr. Yi Ai and Dr. Jinlu Zhang; I appreciate them not only for their excellent technical help and constructive suggestions, but also for being good listeners and supporters. I am very grateful for the excellent job of Stewart Surgener and Robert Slevin on their work on HPLC and electron microscopy. I am also indebted to Qing Cai, Aaron Loveland and Eric Forman for their lending a hand to me on their expert areas, being electron microscopy, statistics, and animal care, respectively. I have also profound gratitude to Dr. Zhiming Zhang and Dr. Richard Grondin for their continuous input and suggestions on my research. There is a big and cooperative staff team in our lab; I owe the happy and relaxing environment to them all: Sheila McLain, Tracy Barber, Liya Liu, Ashley Walton, Mike Dunlap, Naomi Carter and Amy Branham.

I also want to express my gratitude to Ms. Cynthia Long from the Histology facility and Dr. Bruce Maley, Ms. Mary Gail Engle and Ms. Mary Jennes from the Electron Microscopy and Imaging Facility for their excellent help and expertise in the work of paraffin-embedding, confocal microscopy and electron microscopy. I also owe much thanks to Dr. Bruce Maley for his direction on the process of electron microscopy work.

It's hard to stay in the United States away from my loving family for a long period of time. But I know that my strength to continue my work came from my parents in China. The Pacific Ocean does not change our intimacy and connection in any way, and my parents are always the strongest supporters of my work and personal life.

It is impossible to end my acknowledgements without mentioning the graduate program in the Department of Anatomy and Neurobiology and the many faculty and staff I have interacted with during my graduate school training at the University of Kentucky; I appreciate very much all of their support and help.

TABLE OF CONTENTS

Acknowledgements.....	iii
List of Tables	ix
List of Figures.....	x
Chapter One: Effects of Glial Cell Line-Derived Neurotrophic Factor (GDNF) on Stem/progenitor Cell Proliferation and Differentiation	1
Glossary	1
Background.....	3
Stem cells in the central nervous system (CNS).....	3
Adult cell genesis - neurogenesis and gliogenesis.....	5
Neurotrophic factors	6
Neurotrophic factors and cell genesis.....	8
GDNF and its receptors	9
Function of GDNF in the brain.....	11
Effects of GDNF on learning and memory.....	12
Effects of GDNF on neurogenesis	13
Experimental Design.....	15
Concluding Remarks.....	16
Chapter Two: Continuous Intrastratial Infusion of GDNF Increases Cell Genesis in the Striatum, Hippocampus and Substantia Nigra	20
Summary	20
Background.....	21
TGF α and its receptor	21
Effects of TGF α on neurogenesis	22
Cell markers	23
Materials and Methods.....	25
Animals	25
Anesthetization	26
Drug continuous infusion.....	26
BrdU injection.....	27
Tissue collection for infusion studies	27
Immunocytochemistry (ICC)	27
Bioquant.....	28
Statistical analysis.....	30
Immunofluorescent staining.....	31

Results.....	31
GDNF labeling.....	31
Nestin labeling in the brain.....	32
TH labeling in the substantia nigra.....	33
BrdU labeling in the striatum, hippocampus and substantia nigra.....	33
Existence of neurogenesis with traditional double- labeling ICC.....	34
Existence of neurogenesis with immunofluorescent study.....	34
Discussion.....	35
Anesthetization.....	37
The limitation of cell markers.....	37
Bilateral effects of GDNF on stem/progenitor cell proliferation.....	39
The identity of the BrdU positive cells.....	40
 Chapter Three: Morphology of BrdU Positive Cells in Normal Animals with Continuous <i>i.c.v.</i> Infusion of BrdU.....	56
Summary.....	56
Background.....	57
BrdU infusion vs. BrdU injection.....	57
The advantage of 3,3',5,5'-tetramethylbenzidine (TMB) as a chromogen in detecting BrdU labeling.....	57
Materials and Methods.....	58
Animals.....	58
BrdU infusion.....	58
Electron microscopy.....	59
Immunofluorescent staining.....	60
Results.....	60
No evidence of neurogenesis was observed in the substantia nigra.....	60
The phenotype of BrdU labeled cells in different areas of the brain under the electron microscope.....	61
Discussion.....	62
Limitations of the confocal study.....	63
Astrocytes might serve as stem/progenitor cells in the CNS.....	63

Chapter Four: The Effects of Intrastriatal Infusion of GDNF One Month after 6-OHDA MFB Lesion on Stem Cell Proliferation and Differentiation in the Striatum, Hippocampus and Substantia Nigra	68
Summary	68
Background	69
Parkinson's disease (PD)	69
GDNF as a promising drug in the treatment of PD	72
MFB lesion with 6-OHDA	73
Apomorphine-induced rotation test	74
Effects of GDNF on damaged dopaminergic neurons	75
Cell genesis in response to injury	76
Materials and Methods	78
MFB Lesion	78
Apomorphine-induced rotation tests	78
Drug continuous infusion	79
BrdU injection	80
Tissue collection	80
Immunocytochemistry	80
Bioquant	80
Statistical analysis	81
Immunofluorescent staining	81
Results	82
BrdU labeling in the striatum, hippocampus and substantia nigra	82
No significant functional recovery was observed under continuous GDNF treatment in the striatum	83
No neurogenesis in the substantia nigra was observed under GDNF treatment	83
Discussion	84
The MFB lesion model	85
The lack of functional recovery with GDNF treatment one month after MFB lesion	86
Effects of GDNF on stem/progenitor cell proliferation	86
The lack of neurogenesis in the substantia nigra with GDNF treatment in response to injury	87

Chapter Five: The Effects of a Single Intranigral Injection of GDNF Six Hours Before 6-OHDA Partial Lesion on Stem Cell Proliferation and Differentiation in the Striatum and substantia nigra	94
Summary	94
Background	94
The 6-OHDA partial lesion.....	95
GDNF protective effects on dopaminergic neurons	95
High performance liquid chromatography (HPLC) on dopaminergic system	95
Materials and Methods.....	96
Animals and anesthetization	96
GDNF and 6-OHDA injections	96
BrdU injection.....	97
Tissue collection for ICC	97
ICC and immunofluorescent staining	98
Tissue collection for HPLC	98
HPLC	98
Statistical analysis	99
Results.....	99
HPLC study confirmed protective effects of GDNF on dopaminergic neurons.....	99
No neurogenesis in the substantia nigra was observed under GDNF treatment	100
Discussion	100
Chapter Six: General discussion and conclusion	108
References.....	114
VITA.....	142

List of Tables

Table 2.1: Antibodies used in study.....	42
Table 2.2: BrdU+ cell counts in the substantia nigra.....	43
Table 4.1: Procedures undertaken for GDNF/vehicle treatment	88
Table 4.2: BrdU+ cell counts in the substantia nigra.....	89

List of Figures

Figure 1.1: Procedures of GDNF binding to RET.....	19
Figure 2.1: Illustration of osmotic pump implantation	44
Figure 2.2: Stem cells in culture express a series of markers as they differentiate and commit to either neuronal or glial lineages.....	45
Figure 2.3: GDNF labeling	46
Figure 2.4: The number of TH labeled cells in the substantia nigra.....	47
Figure 2.5A: Bioquant cell counting of BrdU labeled cells in the striatum	48
Figure 2.5B: Bioquant cell counting of BrdU labeled cells in the dentate gyrus	49
Figure 2.5C: Bioquant cell counting of BrdU labeled cells in the substantia nigra.	50
Figure 2.6: Confocal microscopy was used to confirm the identity of nestin+ cells.....	51
Figure 2.7: Nestin immunoreactivity in the forebrain	52
Figure 2.8: Nestin immunoreactivity in the hippocampus	53
Figure 2.9: No evidence of neurogenesis in the substantia nigra was observed with traditional double-labeling ICC	54
Figure 2.10: Evidence of neurogenesis in the substantia nigra and hippocampus observed with confocal microscopy.....	55
Figure 3.1: Double-label immunofluorescence staining of BrdU and TH in the substantia nigra of BrdU treated rats	65
Figure 3.2: The morphology of BrdU labeled cells in the dentate gyrus	66
Figure 3.3: The morphology of BrdU labeled cells in the substantia nigra of animals that received continuous BrdU infusion for 28 days	67
Figure 4.1: Rotation behavior tests induced by apomorphine administration after either vehicle or GDNF treatment	90
Figure 4.2A: Bioquant cell counting of BrdU labeled cells in the striatum	91

Figure 4.2B: Bioquant cell counting of BrdU labeled cells in the dentate gyrus	92
Figure 4.2.C.: Bioquant cell counting of BrdU labeled cells in the substantia nigra.....	93
Figure 5.1.A.: HPLC results on dopamine tissue level in the striatum.....	102
Figure 5.1.B.: HPLC results on dopamine tissue level in the substantia nigra.....	103
Figure 5.1.C.: HPLC results on DOPAC tissue level in the striatum	104
Figure 5.1.D.: HPLC results on DOPAC tissue level in the substantia nigra.....	105
Figure 5.1.E.: HPLC results on HVA tissue level in the striatum	106
Figure 5.1.F.: HPLC results on HVA tissue level in the substantia nigra	107

Chapter One

Effects of Glial Cell Line-Derived Neurotrophic Factor (GDNF) on Stem/progenitor Cell Proliferation and Differentiation

Glossary

3-MT	3-methoxytyramine
6-OHDA	6-hydroxydopamine
ABC	avidin: biotin: peroxidase complex
aCSF	artificial cerebrospinal fluid
ANOVA	analysis of variance
ARTN	artemin
ICC	immunocytochemistry
BDNF	brain-derived neurotrophic factor
bFGF	basic fibroblast growth factor
BrdU	5-bromo-2'-deoxyuridine
CNS	central nervous system
CNTF	ciliary neurotrophic factor
COMT	catechol-O-methyltransferase
DAB	diaminobenzidine
DAT	dopamine transporter

DMSO	dimethyl sulfoxide
DOPAC	3,4-dihydroxyphenylacetic acid
EGF	epidermal growth factor
EGFR	EGF receptor
GABA	γ -aminobutyrate acid
GAD	glutamate acid decarboxylase
GDNF	glial cell line-derived neurotrophic factor
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
GFR α	GDNF family receptor α
GPI	glycosylphosphatidylinositol
HPLC	high performance liquid chromatography
HVA	homovanillic acid
ICC	immunocytochemistry
<i>i.c.v.</i>	intracerebroventricular
IGF-1	insulin growth factor-1
<i>i.p.</i>	intraperitoneal
LV	lateral ventricle
MAO	monoamine oxidase
MAP-2	microtubule-associated protein-2
MFB	medial forebrain bundle
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NCAM	neural cell adhesion molecule

NeuN	neuronal nuclei
NGF	nerve growth factor
NSE	neuron specific enolase
NTN	neurturin
PD	Parkinson's disease
PSP	persephin
RT-PCR	reverse transcription polymerase chain reaction
SGZ	subgranular zone
SNpc	substantia nigra pars compacta
SVZ	subventricular zone
TGF α	transforming growth factor α
TGF β	transforming growth factor β
TH	tyrosine hydroxylase
TMB	3,3',5,5'-tetramethylbenzidine
VTA	ventral tegmental area

Background

Stem cells in the central nervous system (CNS)

Stem cells are undifferentiated and unspecialized cells that can divide to reproduce themselves and/or give rise to one or more specialized types of cells. To be considered a stem cell in the CNS, a cell must have multipotency: the potential to differentiate into all three cell lineages including neurons, astrocytes, and

oligodendrocytes. Besides, it should have a capacity for unlimited self-renewal throughout the adulthood and will never differentiate (Weiss et al. 1997). A progenitor cell is, by definition, a cell producing terminally differentiated cells through a limited capacity for self-renewal (McKay, 1997). The term “precursor” is less stringent, referring to any cell that is earlier in a developmental pathway than another. It is often used when it is not clear whether a stem cell or a progenitor cell has been studied. “Plasticity” is referred to as the ability of the adult brain to change its anatomy in response to external or internal stimuli.

Stem cells have two potential mitotic pathways, the symmetric division and the asymmetric division. When a stem cell undergoes symmetric division, both of the progeny will be stem cells. With asymmetric division, a stem cell produces two daughter cells. One is a stem cell whereas the other is a progenitor cell that begins a pathway towards terminal differentiation. Thus, a stem cell has the ability to generate a large number of differentiated cells through an extensive self-renewal and production of progenitor cells.

It was once thought that the capability for stem cells to proliferate and produce different cell types was restricted to the embryonic stage; and recovery from brain injury occurred only by building up new synaptic connections between remaining healthy cells. The scarce proliferation activity within the CNS was attributed solely to the generation of glia, the process called gliogenesis. New evidence shows the existence of multipotent stem cells along the subventricular zone (SVZ, Bayer et al. 1982; Crespo et al. 1986; Reynolds and Weiss, 1992; Richards et al. 1992) and in the subgranular zone (SGZ, Gage et al. 1995; Palmer et al. 1997) of the hippocampus in the adult brain. SVZ is a 2–3 cell

layer-thick region immediately adjacent to the ependymal lining of the lateral ventricle derived from the embryonic germinal zone of the forebrain; it borders the striatum, septum and corpus callosum. SGZ is a thin lamina between the hilar region and the granular cell layer of the dentate gyrus.

The introduction of [^3H] thymidine labeling techniques allowed the discovery of persistent proliferation existing throughout the adulthood in the SVZ and the SGZ (Altman, 1962; Altman & Das, 1965a,b). The use of a thymidine analogue, 5-Bromo-2'-deoxyuridine (BrdU), further confirmed the existence of stem/progenitor cells in these two brain areas.

The cells isolated from many brain areas, including the SVZ, the SGZ, the cortex, the striatum, and the substantia nigra have been shown to be able to expand indefinitely *in vitro* with growth factor such as basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF). With growth factor withdrawn, they could further differentiate into neurons, astrocytes and oligodendrocytes (Reynolds & Weiss, 1992; Richards et al. 1992). When transplanted back into the SVZ or SGZ, where neurogenesis constantly took place, they could further give rise to newly generated functional neurons (Lie et al. 2002).

Adult cell genesis-neurogenesis and gliogenesis

Neurogenesis, the production of new neurons, has only been identified unequivocally in two regions of the CNS: the dentate gyrus of the hippocampus and the olfactory bulb. It is only within the last decade that the existence of neurogenesis in adult brain has been widely accepted. The concept was first introduced by Altman & Das (1962; 1965a,b) in adult rats with [^3H] thymidine autoradiography. But this method has

two shortcomings. One is that it is hard to prove the identity of the mitotic cells as neurons; the other is the difficulty to measure the extent of neurogenesis quantitatively.

With the introduction of techniques like BrdU labeling and green fluorescent protein (GFP) transfection, it is now clear that neurogenesis does exist in certain brain areas. The stem/progenitor cells residing in the SVZ can migrate to the olfactory bulb through the rostral migratory stream and differentiate into interneurons (Lustin, 1993; Lois & Alvarez-Buylla, 1994), and those in the SGZ can migrate to the nearby dentate gyrus and differentiate into the granule cells (Cameron et al. 1993; Kuhn et al. 1996; Palmer et al. 2000; Seri et al. 2001).

Several criteria have been used to identify a functional newly generated neuron. These include being postmitotic, polarized, capable of firing action potentials and able to communicate with other neurons through synapses. Up to date, studies on neurogenesis have relied mostly on morphological or immunohistochemical evidence. There is still a long way to go in investigating the identity and location of newly generated cells.

Neurotrophic factors

Neurotrophic factors, by definition, are endogenous soluble proteins that promote the long term survival, differentiation and maintenance of neurons (Barde, 1989; Gotz and Scharf, 1994). It is now generally accepted that they are signaling molecules important for the development and maintenance of structural integrity within the peripheral and central nervous systems. They are generally small, soluble proteins with molecular weights between 13 and 24 KDa and are often active as homodimers.

Like most signaling molecules, neurotrophic factors can be divided into families formed of closely related molecules. They are produced by both glial cells and neurons. A prototypic neurotrophic factor, such as nerve growth factor (NGF), is a target-derived molecule that binds to a transmembrane receptor on the cell surface (Barbacid 1995; Segal and Greenberg, 1996). The receptor then dimerizes and is activated by transphosphorylation of the catalytic intracellular domain, which starts a complex intracellular signaling pathway leading to immediate, early and late transcriptional changes in the target cell. Some of the neurotrophic factors, such as the ciliary neurotrophic factor (CNTF), are also secreted, but not derived from a distant target tissue. These molecules are produced in an autocrine or paracrine fashion and exert their function on neuronal cells. Some other neurotrophic factors, including integrins, are not secreted and have initially been considered as adhesion molecules. They are now found to have growth-promoting functions, such as mediating cell differentiation.

It is well known that excess neurons are generated during neural development. The survival and differentiation of an appropriate number of neurons is largely regulated by target-derived neurotrophic factors. Developing neurons that fail to compete and make connections with their targets will be deprived of necessary neurotrophic factors and die. Those neurons that establish connections survive and function properly.

Neurotrophic factors are also capable of promoting the re-growth of damaged neurons and their processes both *in vitro* and in animal models. Thus, they represent exciting possibilities for reversing devastating brain disorders, including Parkinson's disease (PD).

Neurotrophic factors and cell genesis

Neurotrophic factors are also known to increase adult neurogenesis (Goldman, 1998). They are endogenous soluble small proteins that promote the survival and maintenance of neurons. Below are several examples of their involvement in neurogenesis.

(1) Intracerebroventricular (*i.c.v.*) injections of EGF increased the proliferation of stem/progenitor cells in the SVZ and lead to differentiation of the stem cells into glia and the reduction of newborn neurons in the hippocampus and the olfactory bulb (Craig et al. 1996; Kuhn et al. 1997). EGF also maintained and stimulated proliferation of adult stem cells *in vitro* (Reymolds & Weiss, 1992).

(2) FGF stimulated the proliferation of neural stem cells and promoted neuronal production in many brain regions including the olfactory bulb (Kuhn et al. 1997). It could stimulate adult stem cell proliferation and maintain them *in vitro* (Richards et al. 1992).

(3) Brain-derived neurotrophic factor (BDNF) administration has been shown to induce neurogenesis in the striatum, septum, thalamus, hypothalamus, and olfactory bulb (Zigova et al. 1998; Benraiss et al. 2001; Pencea et al. 2001).

(4) Insulin growth factor -1 (IGF-1) promoted the proliferation of stem cells and support cell survival (Anderson et al. 2002).

(5) Transforming growth factor α (TGF α) administration, may promote stem cell proliferation, migration and differentiation into dopaminergic neurons in the striatum (Fallon et al. 2000), but the results are controversial (Cooper & Isacson, 2004; Frielingsdorf et al. 2004).

GDNF and its receptors

GDNF (Lin et al., 1993) and the related proteins neurturin (NTN) (Kotzbauer et al., 1996), persephin (PSP) (Milbrandt et al., 1998) and artemin (ARTN) (Baloh et al., 1998) define a novel family of neurotrophic factors that is a sub-group of the transforming growth factor β (TGF β) superfamily.

Unlike other members of the TGF β superfamily, which signal through the receptor serine-threonine kinases, GDNF family ligands activate intracellular signaling cascades via receptor tyrosine kinase. The receptors of the GDNF family ligands have multiple components. They include a signaling unit, the membrane-spanning receptor tyrosine kinase RET (Durbec et al. 1996; Trupp et al. 1996), and a high-affinity ligand binding protein, the GDNF family receptor α (GFR α , Jing et al. 1996; Treanor et al. 1996). As depicted in [Fig. 1.1.](#), GDNF family ligands first bind to the glycosylphosphatidylinositol (GPI)-anchored GFR α , and then the GDNF family ligand–GFR α complex binds to and stimulates autophosphorylation of RET (Trupp et al. 1998; Rosenthal, 1999). Alternatively, a pre-associated complex between GFR α and RET could form the binding site for the GDNF family ligand (Eketjall et al. 1999). GFR α 1, GFR α 2, GFR α 3 and GFR α 4 are the physiological co-receptors for GDNF, NTN, ARTN and PSP, respectively (Airaksinen & Saarma, 2002).

The first member of this family, GDNF, is a glycosylated and disulfide-bound homodimer. The biologically active form of GDNF is composed of two 134 amino acid monomers that migrate in gels with an apparent molecular weight in the 33-45 kDa range. Although all GDNF family ligands could signal via activated RET, GDNF can also signal via GFR α 1 in the absence of RET (Trupp et al. 1998). GPI-anchored GFR α

receptors are localized in plasma membrane to lipid rafts. GDNF binding to GFR α 1 also recruits RET to the lipid rafts and triggers association with Src, which is required for effective downstream signaling, leading to differentiation and neuronal survival.

Alternatively, a multicomponent receptor system consisting of GFR α -1 and neural cell adhesion molecule (NCAM, Paratcha et al., 2003) could be activated by GDNF. NCAM is abundantly expressed in Schwann cells and the hippocampal and cortical neurons (Crossin & Krushel, 2000). NCAM-deficient mice displayed structural abnormalities in the rostral migratory bulb, the olfactory bulb, and the hippocampus, as well as the functional deficits in learning and memory (Tomasiewicz et al. 1993; Cremer et al. 1994; Cremer et al. 1997; Chazal et al. 2000). This suggests that GDNF may utilize NCAM signaling pathways to promote axonal growth in hippocampal and cortical neurons in a RET-independent way. A recent publication by Enomoto et al (2004) challenged this idea with the use of specific transgenic mice that express GFR α 1 under a RET promoter on a GFR α 1 null background. Although these mice lack all RET-independent GFR α 1 expression, no structural abnormalities in the olfactory bulb and the hippocampus were detected.

The mRNA expression of GDNF and its receptor has been identified in many tissues and species by a variety of techniques, including *in situ* hybridization (Hellmich et al., 1996; Suvanto et al., 1996), RNase protection (Trupp et al., 1995), and reverse transcription polymerase chain reaction (RT-PCR) (Choi-Lundberg and Bohn, 1995). GDNF was found to be first expressed in the rostral part of the mouse neural plate at the embryonic day 7.5 and then in the anterior neuroectoderm until embryonic day 10.5 (Hellmich et al., 1996; Suvanto et al., 1996). From the late embryonic stage until

adulthood, the GDNF mRNA expression could be seen in the target areas for nigral dopamine neurons including the caudate, putamen, globus pallidus, and nucleus accumbens (Choi-Lundberg and Bohn, 1995; Trupp et al., 1997). GDNF mRNA could also be detected in ventromedial and ventrolateral thalamic nuclei at high levels and throughout the hippocampus at low levels. Several neuronal populations responsive to GDNF express RET. These include dopaminergic neurons in the substantia nigra and spinal motoneurons. On the other hand, mRNA of GFR α -1 is expressed in many more areas of the brain. A high level of GFR α -1 mRNA is expressed in both dopaminergic and GABAergic neurons in the substantia nigra (Sarabi et al. 2001). GFR α -1 mRNA expression is also found in the GABAergic neurons in the cortex, hippocampus, reticular thalamic nucleus and septum as well as by cells in the cerebellum and motoneurons in the spinal cord (Sarabi et al., 2000, 2003). This suggests that GDNF can effect many different cell populations in the brain, including nigral dopaminergic and GABAergic neurons.

Function of GDNF in the brain

GDNF family ligands are potent survival factors for midbrain dopaminergic neurons, as well as for other forms of neurons. Early studies on GDNF provided strong evidence that it is essential for the survival, differentiation and high-affinity dopamine uptake of midbrain dopaminergic neurons *in vivo* and *in vitro* (Lin et al., 1993). GDNF specifically affects cultured dopaminergic midbrain neurons without affecting other neurons or glia of the substantia nigra (Lin, 1996). It increases dopaminergic cell number, dopamine uptake, cell size and axon sprouting (Hudson et al., 1995; Ai et al. 2003)

without any change in γ -aminobutyrate acid (GABA) or serotonin uptake or overall number of neurons.

With the use of GDNF knock-out mice and transgenic mice, Granholm et al (2000) found that postnatal development of dopaminergic neurons was severely disturbed as a result of the GDNF null mutation. This suggests that GDNF is essential for postnatal survival and/or TH expression of ventral mesencephalic dopaminergic neurons.

GDNF was also found to have trophic effects on many other populations of neurons *in vivo* and *in vitro*, including spinal motoneurons (Henderson et al., 1994; Oppenheim et al., 1995; Yan et al., 1995), central noradrenergic neurons (Arenas et al., 1995), cerebellar Purkinje cells (Mount et al. 1995), and peripheral neurons (Buj-Bello et al., 1995; Trupp et al., 1995). GDNF is also required for promoting ureteric branching in kidney development and regulating spermatogenesis.

GDNF has also been shown to act as a target-derived factor in the olfactory system and may prolong the lifespan of the receptor neurons, hence allowing them time for differentiation (Buckland & Cunningham, 1999).

Effects of GDNF on learning and memory

GDNF is a potent trophic factor for midbrain dopaminergic neurons innervating the hippocampus (Ishikawa et al. 1982). Thus, GDNF may promote neuronal survival and enhance plasticity in hippocampal neurons (Lindvall et al. 1994). Upon insult to the brain, there was evidence of activity-dependent alterations in GDNF mRNA expression within the hippocampus (Reeben et al. 1998; Kokaia et al. 1999). An *i.c.v.* administration of GDNF improved spatial learning in aged rats (Pelleymounter et al. 1999).

It has also been reported (Gerlai et al. 2001) that heterozygous GDNF mutant mice demonstrated significant and selective impairment of performance in the Morris water maze without detectable blood chemical and pathological changes. The Morris water maze test is a widely used spatial task of learning and memory that mainly involves the hippocampus. This suggested that endogenous GDNF may play a critical role in cognitive function.

The noradrenergic locus coeruleus-hippocampal pathway is important in the process of learning and memory. GDNF is a potent neurotrophic factor on locus coeruleus noradrenergic neurons. By using wild type or GDNF knockout fetus brain transplantation, Quintero et al. (2004) have unequivocally shown that the normal development of this pathway was disrupted by GDNF null mutation. This also suggests that GDNF may play an essential role in learning and memory.

Up to now, there has been no published report on the mechanism through which GDNF could exert its function on the brain regions related to learning and memory, especially in the hippocampus.

Effects of GDNF on neurogenesis

As a potent trophic factor for dopaminergic neurons, GDNF exerts its function through targeting the nigrostriatal system, especially the striatum and the substantia nigra. Both of these structures have endogenous stem/progenitor cells. Stem/progenitor cells were observed and isolated from the adult striatum with maintained proliferative activity (Palmer et al. 1995; Kuhn et al. 1997; Mao et al. 2001). The addition of new neurons has been shown in the adult striatum in normal squirrel monkeys (Bedard et al. 2002) and in

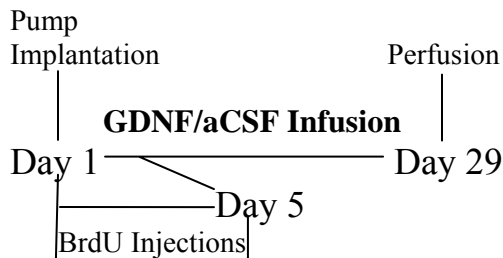
rodents under neurotrophic factor treatments (Craig et al. 1996; Fallon et al. 2000; Benraiss et al. 2001; Pencea et al. 2001). These new neurons might be derived from the endogenous striatal progenitor cells or from the neighboring stem/progenitor cells in the SVZ. There is recent evidence that dopaminergic neurites exist in close contact with stem/progenitor cells in the SVZ (Hoglinger et al. 2004), suggesting a possible role of GDNF on these cells. Endogenous stem/progenitor cells were also observed and isolated from the substantia nigra. They had the ability to differentiate into glial cells *in vivo* and to produce all cell lineages *in vitro* (Lie et al. 2002). When transplanted back into the hippocampus, these stem/progenitor cells could survive and differentiate into neurons, suggesting an important role of the local environment in promoting neurogenesis. These cells may also be responsive to GDNF treatment, which could serve as a proneuronal signal.

Although GDNF levels decrease markedly after development, the endogenous level of GDNF could increase after certain experiences, such as an enriched environment (Young et al. 1999). Dopaminergic fibers have been identified in the SGZ of the hippocampus, and dopaminergic denervation caused the loss of these fibers in mice (Hoglinger et al. 2004). These reports suggest that hippocampal neurogenesis may be partly under dopaminergic control. Since exposure to an enriched environment has been shown to promote survival of newly generated neurons in the dentate gyrus (Kempermann et al. 1997), it is possible that GDNF could play a role in hippocampal neurogenesis. Could GDNF serve as a proneuronal signal and induce neurogenesis in the striatum, the hippocampus, and the substantia nigra? We aim to answer these questions in the following experiments.

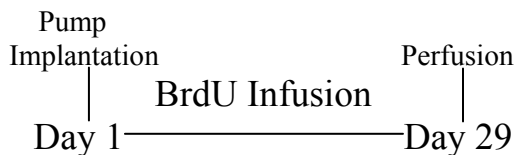
Experimental Design

In this research project, we designed a series of experiments to answer some of the following questions: a) Will GDNF continuous intrastriatal infusion be sufficient to induce neurogenesis in normal animals? b) What is the morphology of the newly generated cells in the brain? c) Will GDNF continuous intrastriatal infusion improve motor function of animals with severe 6-OHDA lesion and induce neurogenesis in the brain besides its restorative effects? d) Will GDNF intranigral injection induce neurogenesis in the substantia nigra in addition to protecting dopaminergic neurons from 6-OHDA partial lesion?

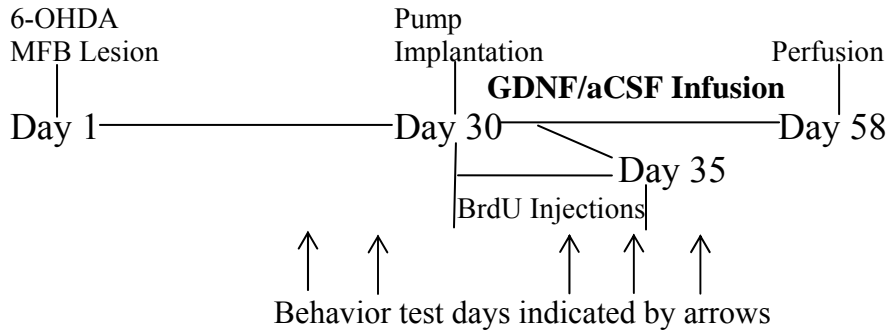
Study 1: Effects of GDNF continuous infusion into the striatum of normal rats (n=9)



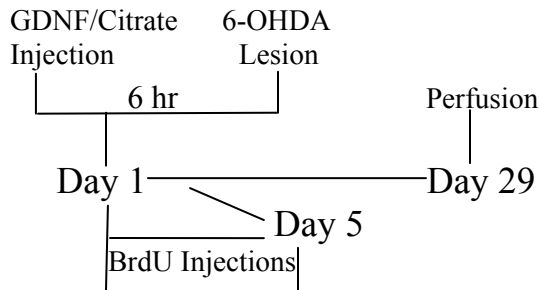
Study 2: Characteristics of BrdU labeled cells with BrdU continuous infusion into the lateral ventricle of normal rats (n=6 for electron microscopy; n=4 for immunofluorescent study)



Study 3: Effects of GDNF continuous infusion into the striatum of 6-hydroxydopamine (6-OHDA)-lesioned rats (n=9)



Study 4: Effects of GDNF on rat brain when delivered in the substantia nigra six hours before a 6-OHDA lesion (n=8)



Concluding Remarks

The focus of this dissertation research has been to investigate the effect of GDNF on stem/progenitor cell proliferation and differentiation during the adulthood. Because GDNF is a potent trophic factor for dopaminergic neurons and it may play a role in learning and memory, we focus our research on the basal ganglia and the hippocampus.

The main hypotheses were tested in four specific aims:

1. Continuous striatal infusion of GDNF/TGF α will induce stem/progenitor cells to proliferate and differentiate into cells expressing neuronal marker proteins, confirming and extending the findings of Fallon et al (2000).

2. Delivery of BrdU through continuous *i.c.v.* infusion will label more stem/progenitor cells than through repeated *i.p.* injection; this technique will allow the detection of newly generated neurons in the basal ganglia. This work is to confirm and extend the work by Zhao et al (2003).
3. Continuous infusion of GDNF into the striatum of rats with unilateral 6-OHDA medial forebrain bundle (MFB) lesions will improve motor function and induce stem/progenitor cells to proliferate and differentiate into cells expressing neuronal marker proteins.
4. A single intranigral injection of GDNF into the rat brain six hours before a unilateral 6-OHDA lesion will protect dopaminergic neurons against the neurotoxin, increase the tissue level of dopamine and its metabolites, and induce stem/progenitor cells to proliferate and differentiate into cells expressing neuronal marker proteins. This is to confirm and extend the results from Kearns et al (1997).

The data will be presented from [Chapter 2](#) to [Chapter 5](#) in this thesis. In [Chapter 2](#), we first demonstrated that continuous infusion of GDNF could induce stem/progenitor cell proliferation and migration in the basal ganglia and the dentate gyrus, but not neurogenesis in the substantia nigra. The techniques we used were ICC and immunofluorescence. In [Chapter 3](#), we used immunofluorescent confocal microscopy and electron microscopy techniques to investigate the identity of the BrdU positive cells with BrdU continuous infusion, and concluded that the cells labeled with BrdU were immature and had characteristics of an astrocyte. Apomorphine-induced rotation tests were used

and the results showed no significant functional recovery with GDNF treatment in this scenario. In [Chapter 4](#), a unilateral 6-OHDA MFB lesion was first introduced to animals one month before the GDNF treatment, thus wiping out more than 95% of the dopaminergic neurons in the ipsilateral substantia nigra. We found that GDNF continuous infusion could induce stem/progenitor cell proliferation, but not neurogenesis, in the substantia nigra. In addition to ICC, we used an apomorphine-induced rotation test to monitor the recovery of motor function in trophic factor recipients. In [Chapter 5](#), a single GDNF intranigral injection was administered to rat brain prior to a less severe lesion in the substantia nigra, but still failed to induce neurogenesis in the substantia nigra. High performance liquid chromatography (HPLC) was used to confirm the increased tissue level of dopamine and its metabolites in the substantia nigra with GDNF treatment.

In summary, our findings provided evidence that GDNF induced stem/progenitor cell proliferation in the basal ganglia and hippocampus. No neurogenesis could be observed in the substantia nigra under the GDNF treatment, even with lesion. On the other hand, GDNF may play a role in promoting neurogenesis in the dentate gyrus, where neurogenesis constantly occurs, with or without injury.

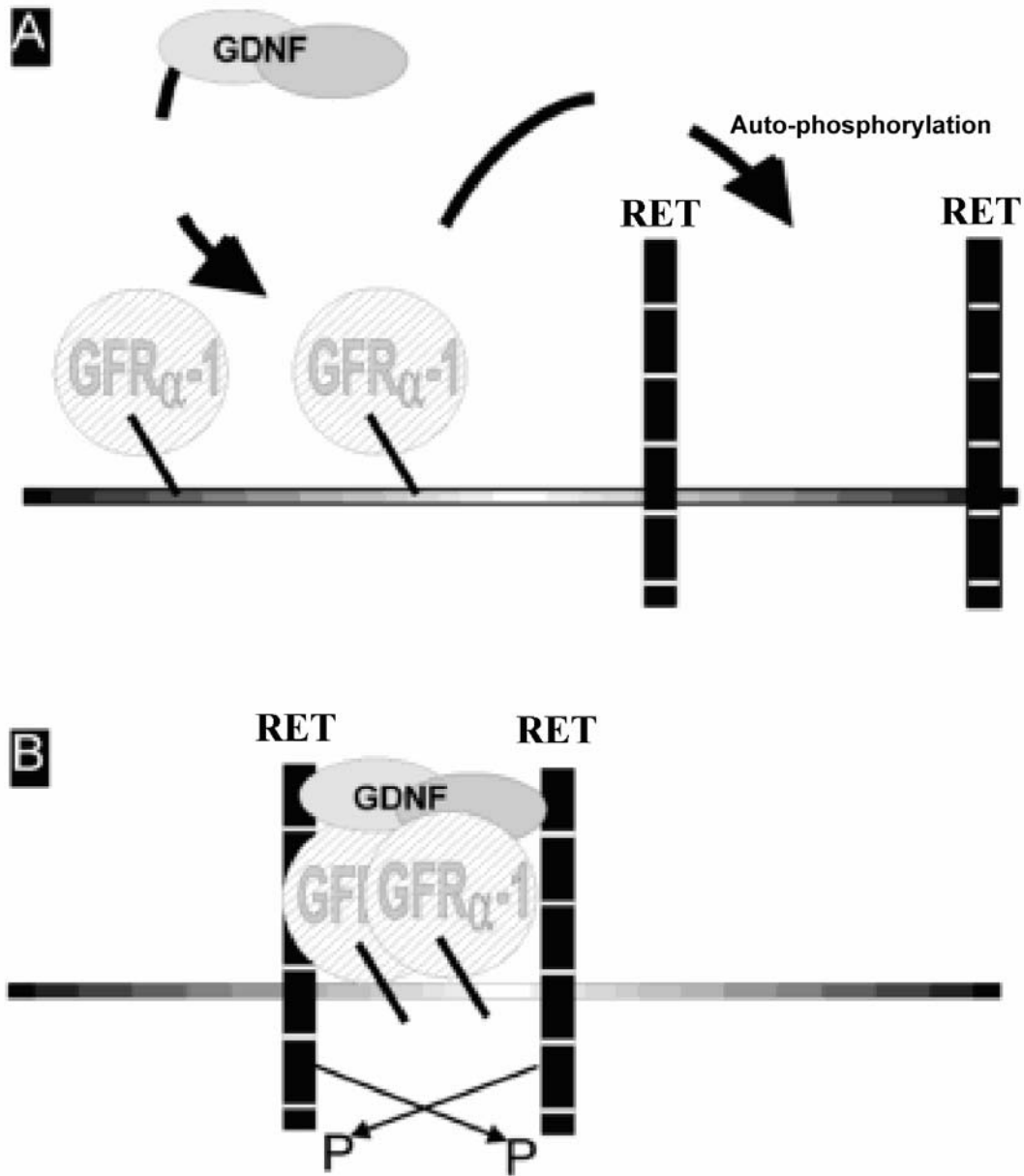


Figure 1.1 – Procedures of GDNF binding to RET. A GDNF dimer first binds to two $GFR_{\alpha-1}$ molecules to form a ligand-receptor complex (A). This complex then binds to two RET molecules, which dimerizes and undergoes antophosphorylation of the tyrosine kinase domain in each subunit (B). Upon this, the RET dimer is activated and starts the downstream signaling pathway.

Figure modified from p18 of the thesis entitled “GDNF and p75 neurotrophin receptor in development and disease” by Wartiovaara K, 1998.

Chapter Two

Continuous Intrastratial Infusion of GDNF Increases Cell Genesis in the Striatum, Hippocampus and Substantia Nigra

Summary

The purpose of this study was to analyze the effects of intrastratial GDNF and TGF α administration on neurogenesis in the striatum, hippocampus and substantia nigra. The results were analyzed to assess the capability of continuous GDNF/TGF α infusion to activate endogenous adult stem cells and to stimulate their proliferation and differentiation into mature glia and neurons. Using Alzet minipumps (model 2004, Durect, Cupertino, CA), the control group of nine rats received vehicle (artificial cerebrospinal fluid, aCSF) infusion into the right striatum at a rate of 0.5 μ l/hr for 28 days (Fig. 2.1.). The two trophic factor recipient groups received either recombinant-Methionyl human GDNF (1.5 μ g/ μ l, Amgen, Thousand Oaks, CA) or TGF α (0.5 μ g/ μ l, PeproTech Inc., Rocky Hill, NJ) at the same flow rate for the same time period with nine animals (n=9) in each group. To label mitotic cells, all animals received intraperitoneal (*i.p.*) injections of 200 mg/kg 5-Bromo-2'-deoxyuridine (BrdU, Sigma, St. Louis, MO) for the first five days of intrastratial infusion. After 28 days of infusion with the trophic factor recipients receiving a total of 600 μ g GDNF or 200 μ g TGF α , the animals were euthanized and their brains recovered for immunocytochemical (ICC) analysis at the light microscopic level (Table 2.1.; Fig. 2.2.). The spread of GDNF in brain parenchyma was evaluated by GDNF ICC (Fig. 2.3.). Unbiased stereological cell counting procedures

were used to quantify the number of tyrosine hydroxylase (TH) positive neurons in the substantia nigra and to verify the effects of GDNF on dopamine neurons (Fig. 2.4.). The intensity of BrdU labeling in the striatum (Fig. 2.5.A) and the number of BrdU positive cells in the dentate gyrus (Fig. 2.5.B) and substantia nigra (Fig. 2.5.C) were counted in every sixth section of the whole brain set. The number of cells double-labeled for BrdU and TH was counted in the substantia nigra (Table 2.2.). The presence of double-labeled cells was verified by confocal microscopy.

While we found a significant increase in the number of dopaminergic neurons in the substantia nigra, and increased cell genesis in all of the three areas examined, as manifested by nestin and BrdU labeling (Figs. 2.6.-2.8.), none of the new cells were labeled with neuronal markers. Thus, with traditional double-labeling ICC and confocal microscopy techniques, we did not observe any newly generated dopaminergic neurons in the substantia nigra (Figs. 2.9.-2.10.).

Background

TGF α and its receptor

TGF α is a member of the EGF family. It binds to the EGF receptor, a tyrosine kinase receptor encoded by the Erb B gene (Todaro et al. 1980). Postnatally, EGF receptor continues to be expressed in regions undergoing active neurogenesis including the cerebellar granule layer, the SVZ, and the granule layer of the dentate gyrus. In the adult rat, EGF receptor expression is restricted to the SVZ and the dentate gyrus (Seroogy et al., 1995; Okano et al., 1996; Kornblum et al. 1997; Doetsch et al. 2002).

Effects of TGF α on neurogenesis

With the use of TGF α null mutants (Tropepe et al. 1997; Conover et al. 2000), this growth factor was found to be crucial for the full proliferation of stem/progenitor cells in the dorsolateral corner of the SVZ and those destined to the olfactory bulb. A separate report showed that TGF α null mice had fewer dopaminergic neurons in the substantia nigra pars compacta (SNpc) prenatally, suggesting a possible mitogenic role of TGF α on the nigrostriatal system (Blum, 1998).

Fallon et al. (2000) reported that continuous intrastriatal infusion of TGF α alone, combined with a 6-OHDA partial lesion, could induce stem cells to proliferate in the SVZ, to migrate into the striatum and to differentiate into dopaminergic neurons. A normalization of rotation behavior in lesioned rats after TGF α treatment was also observed. With or without 6-OHDA lesion, a significant increase in the expression of EGF receptor mRNA in the SVZ during drug infusion was observed. With TGF α treatment, newly generated dopaminergic neurons, which were double labeled with BrdU and dopamine transporter (DAT), were observed in the striatum. This report was the first to show evidence of the induction of endogenous neurogenesis in the dopaminergic system. Recently, however, Cooper & Isacson (2004) and Frielingsdorf et al. (2004) reported independently that they failed to replicate Fallon's results and found no neurogenesis in the basal ganglia.

Since the addition of new dopaminergic neurons in the adult striatum may enhance the nigrostriatal dopaminergic pathway and imply an effective treatment for neurodegenerative disease (as we will discuss in later chapters), it is important to follow Fallon's study to confirm the existence of striatal neurogenesis. GDNF is a potent trophic

factor for midbrain dopaminergic neurons, so it is reasonable to test the response of stem/progenitor cells to the treatment with GDNF in addition to TGF α .

Cell markers

ICC was used to quantify cell genesis, survival and differentiation. Several cell specific markers were used, as described below:

(1) Markers for stem / progenitor cell:

It is essential to have unique markers for identifying stem cells in the study of neurogenesis. The difficulty lies in that the most primitive cells do not express many antigens on the surface or even internally.

BrdU is a synthetic thymidine analogue, which can only be taken up by cells during the S-phase of mitosis, just before mitotic separation, and is a marker of proliferating cells and their progeny.

Nestin is an intermediate filament transiently expressed during neural ontogeny, which is first expressed by neuroepithelial cells and radial glia in development, then by progenitor cells of the ventricular zone later during the embryonic stage (Lendahl et al., 1990). *In vitro* studies show that nestin is only expressed in brain cells with progenitor cell qualities. Nestin has been used as the stem cell marker by many groups, and ICC for nestin in combination with β -III tubulin can be used to label the neuronal progenitor cells.

(2) Markers for immature cells

β -III tubulin (commercially known as Tuj-1) is a composite of the microtubules expressed very early in the process of neuronal differentiation. It is supposed to have a

unique role during early neuronal differentiation and neurite outgrowth, and is used as a marker for neuronal-lineaged cells and embryonic neurons immediately postmitotic.

Microtubule-associated protein-2 (MAP-2) is involved in the microtubule reorganization that accompanies neuronal process elaboration. It selectively stains neuronal cell bodies and dendrites, serving as a more differentiated neuronal marker than β -III tubulin.

Antibody against NG2 can recognize chondroitin sulphate proteoglycan NG2 on a new glial cell type. These cells may morphologically and topographically resemble non-myelinating oligodendrocytes and/or microglia in the adult CNS (Nishiyama et al. 1999). NG2 has recently been used to label immature oligodendrocytes.

(3) Markers for differentiated cells:

Glial fibrillary acidic protein (GFAP), an intermediate filament, is commonly used as the marker for astrocytes.

Antibody against oligodendrocytes could be used to label mature oligodendrocytes.

Ox-42 is a CR3 complement receptor; it could be used as a marker for CNS microglia.

NeuN is a transcription factor that is expressed in the nucleus and cytoplasm of postmitotic neurons. Enolases are cytoplasmic glycolytic enzymes. The γ -enolase, one isoform of the enzyme, is also called neuron specific enolase (NSE) and is expressed only in neurons and neuron-derived cells. So both NeuN and NSE can be used as neuronal markers.

TH catalyzes the reaction converting tyrosine to levo-dopa, the rate-limiting step of the neurotransmitter biosynthesis. TH is widely used to stain monoamine-containing neurons and as a good marker for dopaminergic neurons in the substantia nigra. DAT rapidly takes up extracellular dopamine into presynaptic terminals after dopamine release, and is also used as a marker for dopaminergic neurons.

GABA: a marker for the GABAergic (inhibitory) neurons.

GAD65/67: Glutamate Acid Decarboxylase (GAD) 65KDa and 67KDa. It is a marker that labels CNS GABAergic (inhibitory) neurons.

It is difficult to determine a cell's phenotype unequivocally from ICC staining because the intensity of labeling varies from cell to cell and the signal-to-noise ratio can be low due to variable background staining. Thus, multiple markers were used to classify cell types.

Materials and Methods

Animals

Young adult male Sprague-Dawley rats (Harlan, Indianapolis, IN) were used in this study. All procedures were conducted in the Laboratory Animal Facilities of the University of Kentucky, which are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. The University of Kentucky's Animal Care and Use Committee approved all protocols.

Anesthetization

The animals were anesthetized by chloral hydrate (trichloroacetaldehyde monohydrate) at the dosage of 300mg/kg body weight. The concentration of the drug solution was 0.02g/ml.

Drug continuous infusion

Rats were randomly divided into three groups (n=9): one group received GDNF, another received TGF α , and the third group received aCSF. The table top where the experiments were performed, and the stereotaxic instrument, were all disinfected with isopropyl alcohol. The scalp of the anesthetized animal was shaved and the animal placed in the stereotaxic device. A midline sagittal incision about 2.5 cm long was made using sterile instruments and the skull exposed. A subcutaneous pocket in the mid-scapular area of the back of the rat was created with a hemostat. Then the Alzet osmotic minipump (Durect Corporation, Cupertino, CA; model 2002: 200 μ l/14 days) was inserted into the subcutaneous pocket, leading the attached catheter to the site for cannula placement. A small hole was drilled in the skull over the right frontal cortex and a 5 mm cannula attached to the minipump was stereotaxically implanted into the right caudate-putamen (coordinates- AP: 1.2mm, ML: -2.7mm) using Bregma as a reference. The pump was implanted subcutaneously and the infusate (200 μ l GDNF or TGF α or aCSF) was delivered directly into the striatum at a rate of 0.5 μ l/hour. The concentration of GDNF was 1.5 μ g/ μ l and the concentration of TGF α was 0.5 μ g/ μ l. After cannula implantation, the animal was kept warm on a heating pad until awaking, and then placed back into the cage with food and water. It was closely monitored for sign of distress from surgery and

anesthesia. At the 14th day of infusion, the animal was anesthetized and placed back in the stereotaxic device. A small incision was made on the back skin and the pump was replaced by a new one full of the same infusate (200 μ l). Altogether, 600 μ g GDNF or 200 μ g TGF was delivered to trophic factor recipients.

BrdU injection

BrdU (Sigma, St. Louis, MO) was dissolved in 0.9% saline at a concentration of 200mg/15ml, and sterilized with a 0.2 mm filter. Beginning from the first day of infusion, the animal received one *i.p.* injection of freshly made BrdU per day for five days consecutively. The dosage used each time was 200mg/kg.

Tissue collection for infusion studies

At the end (28th day) of drug infusion, the animal was anesthetized with Ketamine and sacrificed by transcardial perfusion of 4% paraformaldehyde. The brain was collected and postfixed overnight, and then it was immersed in 30% sucrose for three days before slicing on a freezing microtome. Samples were sectioned at a thickness of 30 μ m and stored in the cryoprotectant solution at -20°C.

Immunocytochemistry (ICC)

Free-floating sections stored in cryoprotectant were used. They were rinsed in 0.1M PBS, immersed in 0.3% H₂O₂, and blocked by 4% normal goat serum with 0.4% Triton X-100 in PBS before primary antibody incubation for one to two days. Primary antibodies generated in mouse, rat, rabbit recognizing the following antigens were used:

mouse anti-nestin (Rat401; 1:20, Developmental Studies Hybridoma Bank, Iowa City, IA), mouse anti- β -III tubulin (Tuj-1; 1:1,000, Covance, Berkeley, CA), mouse anti-neuronal nuclear antigen (NeuN; 1:500, Chemicon), mouse anti-TH (1:1,500, Chemicon), mouse anti- GFAP (1:1,000, Chemicon), mouse anti-NG2 (1: 200, Chemicon), mouse anti-MAP2 (1:200, Chemicon), mouse anti-GABA (1:200, Chemicon), rat anti-DAT (1:5,000, Chemicon), rabbit anti-tyrosine hydroxylase (TH; 1:2,500, Pel-freeze, Pogens, AR), and mouse anti-OX42 (1:4,000, BD PharMingen, San Diego, CA). The sections were then rinsed in PBS and incubated in biotinylated secondary antibodies. The antigens were visualized by using ABC solution (ABC standard kit, Vector) followed by diaminobenzidine (DAB)-peroxidase histochemistry.

For BrdU ICC, rat anti-BrdU (1:200, Accurate Chemical, Westbury, NY) was used. Pretreatments were needed before the incubation of the primary antibody. DNA was denatured by 50% formamide in 2 \times SSC for two hours at 65°C, and then the sections were rinsed in 2 \times SSC, and incubated in 2N HCl at 37°C for 30 minutes. After being rinsed in PBS, sections were then immersed in 0.1M boric acid (pH 8.5) for 10 minutes, followed by a rinse in PBS.

Bioquant

The Bioquant Image Analysis System was used to quantify dopaminergic neurons in the substantia nigra and the newly generated cells in certain brain areas.

The number of TH-positive neurons in the SNpc was estimated using an optical fractionator method for unbiased stereological cell counting (West et al., 1991; West 1993; Harding et al., 1994). With a random selection of the first section, every sixth

section thereafter was sampled through the entire SNpc, which entailed the analysis of seven to ten sections per animal. The average section thickness was 14 μm , as determined from measuring mounted sections from each animal. The SNpc was defined as consisting of all midbrain TH-positive neurons except those interspersed with the oculomotor nerve rootlets. On each section, a $120\mu\text{m} \times 120\mu\text{m}$ grid was superimposed, with an $80\mu\text{m} \times 80\mu\text{m}$ counting chamber placed on each grid. All of the TH-positive neurons with clearly identified nuclei were counted, if they were either completely within the boundaries of the dissector box or crossing the upper or right side of the box within its 14- μm -depth. This resulted in up to 719 TH-positive cells counted per side per animal. The estimated total number of TH-positive neurons in the substantia nigra was calculated based on the following formula: $N = \Sigma Q- \times 1/\text{ssf} \times 1/\text{asf} \times t/h$ (West et al., 1991) where N is the estimate of the total number of cells, $\Sigma Q-$ is the number of counted cells on the sampled sections through the substantia nigra, ssf is the section sampling fraction, asf is the area sampling fraction. For t/h , t is the actual section thickness and h is the height of the dissector box.

The areas being investigated for BrdU labeling include the caudate-putamen, the dentate gyrus of the hippocampus, and the substantia nigra. One in every 6th of the whole brain sections was used. The sections used for counting cells in the striatum were labeled with BrdU and counter-stained with neutral red. The sections from the hippocampus were double labeled with BrdU and NeuN, and those from the substantia nigra were double labeled with BrdU and TH. The area (number of pixels) of the regions was quantified. The number of BrdU-positive cells in each region was estimated. On each section, a $300\mu\text{m} \times 300\mu\text{m}$ grid was superimposed with a $300\mu\text{m} \times 300\mu\text{m}$ counting chamber placed

on each intersection. All cells completely within the boundaries of the chamber or crossing the upper or right side of the chamber were counted.

The total number of BrdU+/TH+ double-labeled neurons was counted on every sixth section containing the substantia nigra. Similarly, the number of BrdU+/NeuN+ neurons was counted in the granule cell layer of the dentate gyrus of every sixth section.

Statistical analysis

The results are expressed as means \pm SEM. Statistical significance in each side between the treatment groups was assessed using two-way analysis of variance (ANOVA) with side as within-subjects repeated factor and treatment as the between-subjects factor. Subsequently, the effect of GDNF treatment was assessed for each side separately by two-tailed Student's t-tests for independent samples. Unequal variance was assumed. The differences in the number of BrdU+ or TH+ cells in the striatum and the substantia nigra between the vehicle-treated group and the GDNF-treated group were analyzed. For BrdU immunoreactivity in the dentate gyrus, the number of BrdU labeled cell were quantified, and the length of each corresponding dentate gyrus was also measured. The average number of BrdU+ cells in each 100 μ m-long segment of the dentate gyrus was calculated for each section and averaged in each side. The results were compared between the two treatment groups and presented.

Immunofluorescent Staining

Free-floating sections stored in cryoprotectant solution were used. For fluorescent double-labeling, they were first pre-treated, blocked by 4% normal goat serum, and incubated in anti-BrdU primary antibody (as described above) for 48 hours, and rinsed thoroughly and incubated with Alexa Fluor 568 goat anti-rat antibody (1:500, Molecular Probes, Eugene, OR) for one hour. After being rinsed in PBS, the sections were blocked by 4% normal goat serum again before being incubated in another primary antibody (as listed above) for one to two days. Corresponding to the source of the primary antibody applied, Alexa Fluor 488 secondary antibody was used following complete rinsing in PBS. Fluorescent sections were evaluated using Leica TCS laser scanning confocal microscope systems (Am Friedensplatz, Mannheim, Germany).

Results

GDNF labeling

In coronal sections through the region of the striatum containing the catheter, the entire hemisphere of the brain was filled with GDNF in the GDNF recipients. In addition to the striatum, corpus callosum and cortex, this included the right lateral ventricle, suggesting that significant levels of GDNF could have been transported through the CSF. Periventricular GDNF ICC staining was evident through the full extent of the right lateral ventricle and the third ventricle. Some GDNF staining was evident around the ventricular borders of the right hippocampus ([Fig. 2.3A](#)). Retrograde transport of GDNF

was evident in the right substantia nigra, with many neurons displaying GDNF+ staining (Fig. 2.3B, C).

The left hemisphere of the brain was not heavily immunostained for GDNF. Some GDNF immunostaining was present in the contralateral septum, midline cortex and left hypothalamus adjacent to the third ventricle. However, GDNF+ staining was not seen in the left substantia nigra. The left hippocampus was mostly devoid of GDNF immunostaining.

Nestin labeling in the brain

Nestin staining was observed in all of the treatment groups in several brain regions, including the areas lining the lateral ventricle and around the catheter site (Fig. 2.7.). It was also extensive in the hippocampus (Fig. 2.8.).

TGF α recipients had the most extensive nestin immunoreactivity. There was a massive stem/progenitor cell proliferation in the SVZ shown by the increased volume of nestin staining along the lateral ventricle, especially on the infusion side. There was also an S-shaped ridge of cells stained with nestin that originated from the ipsilateral dorsal SVZ, migrated through the corpus callosum, and projected toward the infusion site. All these results were in consistence with those reported by Fallon's group (2000).

When compared with the aCSF (vehicle) treated group, the GDNF recipients had higher intensity of nestin staining in both the SVZ (Fig. 2.7.) and the hippocampus (Fig. 2.8.).

By using double labeling immunofluorescent staining, it was confirmed that a high percentage of the nestin positive cells were stem/progenitor cells that incorporated BrdU as well (Fig. 2.6.).

TH labeling in the substantia nigra

Quantitative cell counting showed a significant increase in TH⁺ neurons in the right substantia nigra of GDNF recipients (Fig. 2.4). The average number of immunopositive neurons per animal was increased in the right nigra by over 30%, from 7010 ± 398 (s.e.m.) in vehicle recipients to 9249 ± 619 in the rats receiving GDNF.

BrdU labeling in the striatum, hippocampus and substantia nigra

The GDNF recipients had a significantly higher number of BrdU labeled cells in the contralateral striatum (Fig. 2.5.A). There is concern that cannula implantation by itself could cause a local cell response and increase the number of BrdU⁺ cells. Our experience to date is that catheter placement into the brain results in only a mild, localized tissue response (Ai et al., 2003; Grondin et al., 2003). As animals from the control group underwent the same procedures, there is an internal control to exclude the treatment effect.

GDNF significantly increased the number of BrdU⁺ cells bilaterally in both the hippocampus (Fig. 2.5.B) and substantia nigra (Fig. 2.5.C). The number of BrdU positive cells in each 100 μ m-long segment of dentate gyrus in GDNF recipients was increased by ~78%, compared to vehicle controls. The increase was not as great in the substantia

nigra, where the number of BrdU+ cells was ~52% larger in the substantia nigra of the trophic factor recipients.

Existence of neurogenesis with traditional double-labeling ICC

By the use of light microscope double-labeling with BrdU and a neuronal marker (NeuN, or NSE, or TH), we evaluated the existence and extent of neurogenesis in the three brain regions. Co-labeling of BrdU with NeuN/NSE was observed in the dentate gyrus of the hippocampus, but not in the striatum and the substantia nigra (Fig. 2.10.). An additional search for BrdU+/TH+ cells in the substantia nigra (Fig. 2.9.) was conducted by counting every BrdU+ cell in every 6th section through the rostral caudal extent of the substantia nigra. While 1549 BrdU+ cells were counted in the 18 rats (controls and GDNF-treated) used in this study, none were TH+ (Table 2.2.).

Existence of neurogenesis with immunofluorescent study

Selected sections were also evaluated by double-label confocal microscopy for other cell markers (Table 2.1) to determine if any of the BrdU+ cells co-labeled with specific antigens for GABAergic neurons, immature neurons, oligodendroglia, astrocytes or microglia.

While there were many BrdU+ cells identified in the striatum and substantia nigra of both vehicle and GDNF recipients, none were TH+ (Fig. 2.10 A-C). In contrast to the striatum and substantia nigra, BrdU+ granule cells were seen in the hippocampus (Fig. 2.10. D-F). A high percentage of BrdU+ cells were also found to be labeled with nestin. This confirmed that our procedures were labeling stem cells in the brain.

With the exception of BrdU⁺/Nestin⁺ cells in the SVZ and BrdU⁺/NeuN⁺ cells in the dentate gyrus, no double-labeled cells were found in this survey in any of the treatment groups. The TGF α treatment, which followed the procedures used by Fallon's group with only minor modifications, failed to induce the striatal neurogenesis as reported in their publication. Our results were consistent with the recent report (Cooper & Isacson, 2004).

Discussion

The present results demonstrated that stem/progenitor cells existed locally within the SVZ, the dentate gyrus and the substantia nigra.

Our experiments with TGF α continuous infusion failed to replicate the profound effects of this trophic factor on stem cell differentiation in the basal ganglia reported by Fallon et al (2000). Since the publication, there has been no report that could duplicate their results with TGF α treatment. In fact, two separate research groups from Harvard University in the United States and Lund University in Sweden (Cooper & Isacson, 2004; Frielingsdorf et al. 2004) have challenged the conclusion. Independently, they repeated Fallon's experiments with small modifications and found no evidence of any dopaminergic neurogenesis in the nigrostriatal system in any treatment group. It is fair to say that neurogenesis is rare, if present at all, in the nigrostriatal system following intrastriatal TGF α infusion.

Although the proliferation of stem/progenitor cells could be induced or enhanced by GDNF continuous striatal infusion, they do not necessarily undergo further differentiation into functional neurons. This holds true in the substantia nigra at least four

weeks after the start of GDNF treatment. Since we observed a significant increase in the number of dopaminergic neurons in the ipsilateral SNpc, we speculate that GDNF may have either increased TH immunoreactivity of existing dopaminergic neurons, or induced Gliogenesis, thus indirectly influencing dopaminergic neurons. At this time, we could not rule out the possibility that GDNF may activate neurogenesis in response to injury/damage. We designed and carried out studies on two different Parkinsonian rat models under the treatment of GDNF in [Chapters 4 and 5](#).

Neurogenesis in the dentate gyrus was confirmed by confocal microscopy with BrdU and NeuN. There was a significant increase of BrdU positive cells in both sides of the dentate gyrus under GDNF treatment. It has been reported that newly generated neurons in mouse hippocampus mature into functional granule cells (van Praag et al. 2002) and that a reduction in neurogenesis coincides with impaired learning and memory of the hippocampal-dependent tasks (Shors et al. 2001). Pelleymounter et al (1999) reported that *i.c.v.* administration of GDNF improved spatial learning in aged rats. It was reasonable to speculate that GDNF might increase neurogenesis in the hippocampus under normal conditions, thus playing an important role in learning and memory. To test this hypothesis, it is important to see whether this kind of stem cell proliferation and differentiation could be linked to the improvement in behavioral tests on cognition. Furthermore, the issue of whether the newly generated neurons contributed directly to improved learning and memory needs to be addressed.

Since BrdU positive cells hardly express any of the cell markers tested in this experiment, it is important to identify the phenotype of these cells with their ultrastructure characteristics, which we observed in the next [chapter](#).

Several other points of discussion are clarified below:

Anesthetization

Chloral hydrate is an anesthetizing drug commonly used in the literature on animals before surgery. With proper dosage (300mg/kg BW) and concentration (0.02g/ml), its use in our lab has been found to be safe and effective. Typically, it provides stable anesthesia in rats for at least an hour, which is an adequate time period for our surgeries. The concentration of chloral hydrate is critical based on the literature and our own experience. When the concentration of solution used is higher than 50 mg/ml, it can cause problems such as adynamic ileus (Fleischman et al. 1977) and peritonitis in rats. On the other hand, chloral hydrate solution at a concentration as low as 20 mg/ml does not cause these problems, nor does it typically induce perceptible stress in rats.

Two other commonly used anesthetic drugs, Ketamine and Pentobarbital, were not chosen for survival surgeries in our studies. This is because both of the drugs have been shown to change the dopamine release in the brain, thereby interfering with our observation in the dopamine system.

The limitation of cell markers

A lot of controversy has been raised about the use of BrdU as a stem/progenitor cell marker based on the fact that BrdU could also be incorporated into the DNA strand during repair process or in cells undergoing apoptosis (Nowakowski & Hayes, 2000; 2002). Another point is that the permanent replacement of thymidine by BrdU on the DNA strand causes cumulative mutations in rapidly expanding cell populations.

Depending on the number of divisions a stem/progenitor cell undergoes before differentiation, the severity of this effect on newly generated cells is varied. If the mutation occurred at an earlier stage of stem cell proliferation, then the mutation could be expected in a higher number of neuronal offspring. On the other hand, the BrdU labeling probably underestimates the number of newly generated cells because BrdU is only available for nuclear incorporation for a few hours after treatment.

Nestin is widely used as a stem cell marker (Lendahl et al., 1990). However, all commercially available antibodies against nestin have a cross-reaction with an endothelial antigen (Palmer et al. 2000). The nestin antibody may also stain for ependymal cells (Garcia-Verdugo et al., 1998) and for radial glial cells in the developing rat CNS. Thus, it might be hard to discriminate the stem/progenitor cells unequivocally.

If the same cells were stained with both BrdU- and nestin- antibodies, there is reasonable confidence that they are in fact stem cells.

Ox-42 could serve as a microglia marker. However, antibody against ox-42 could label most macrophages, dendritic cells, and granulocytes as well.

In this study, we did confocal immunofluorescent staining for both BrdU and nestin on brain sections. A high percentage of BrdU positive cells along the lateral ventricle were also stained with nestin; thus, they could be identified as stem cells.

It has been argued that each of the neuronal markers has its disadvantages. NeuN is generally accepted to be neuron specific, but it can also label other cell types including cells from the adrenal gland and the intermediate lobe of the pituitary gland (Mullen et al., 1992). NSE can label not only neurons but also astrocytes and oligodendrocytes (Deloulme et al., 1996; Sensenbrenner et al., 1997). MAP-2 labels EGF-responsive

precursor cells, a possible stage of astroglial development (Rosser et al. 1997). In this and the following experiments, we used NeuN, NSE and MAP-2 to label neurons. The expression of at least two antigens in a population of adult generated cells is a good indicator of new neurons.

Similarly, both TH and DAT were used to label dopaminergic neurons. In our study, no cell in the nigrostriatal pathway was observed as double-labeled with BrdU and TH/DAT, confirmed the lack of newly generated dopaminergic neurons.

Bilateral effects of GDNF on stem/progenitor cell proliferation

In our experiments, we observed that unilateral delivery of GDNF into the striatum induced stem/progenitor cell proliferation in both sides of the hippocampus and substantia nigra. This was shown by increased intensity of nestin staining and quantity of BrdU labeled cells in these regions. Although it is hard to compare the number of striatal stem/progenitor cells on the infusion side due to the catheter implantation interference, the BrdU labeling of the contralateral striatum increased significantly in the GDNF recipients when compared to the vehicle recipients. All of these results suggest that unilateral infusion of GDNF may have bilateral effects on stem/progenitor cell proliferation.

This is not surprising, given the evidence that GDNF might be carried by CSF in the brain and exert its function on both hemispheres (Fig. 2.3.A). Besides, GDNF delivered to the striatum could be retrogradely transported and/or diffused back to the substantia nigra (Tomac et al. 1995; Kordower et al. 2000). Thus, a unilateral delivery of GDNF may affect both of the brain hemispheres.

This observation is consistent with our previous reports in rhesus monkeys (Gash et al. 1996; Grondin et al. 2002; Grondin et al. 2003). A chronic unilateral *i.c.v.* infusion of 7.5µg GDNF per day for two months, for example, induced bilateral increases of the stimulus-evoked release of dopamine and the basal extracellular levels of dopamine in the substantia nigra of aged rhesus monkeys (Grondin et al. 2003).

The identity of the BrdU positive cells

The co-localization of BrdU and nestin in the SVZ in all of the animals confirmed the existence of endogenous adult stem/progenitor cells (Fig. 2.6.).

Most of the detected BrdU positive cells in the striatum, hippocampus, and the substantia nigra (Fig. 2.10 A, B, C) did not express any of the cell markers we used in the immunofluorescent study. There were instances where a BrdU+ nucleus was closely juxtaposed to a TH+ cell body and could have been mistakenly counted as a positively labeled neuron. In each instance, closer examination of confocal microscopy revealed that the neuronal nucleus was not BrdU+. In contrast to the striatum and substantia nigra, BrdU+ granule cells were seen in the dentate gyrus of the hippocampus (Fig. 2.10.D.-F.), showing that the procedures could label at least some neurons.

The lack of co-localization of BrdU and other cell markers might be due to several of the reasons listed below. First, it could be that a BrdU positive cell expresses a cell marker that was not yet used. Second, the cell might be a progenitor cell not yet committed to any certain lineage or expressing any specific marker. Third, it might be a quiescent cell during development. Last, the cell may remain as an adult stem cell. These

possibilities were addressed using electron microscopy to observe the ultrastructure and identify the phenotype of these BrdU positive cells.

The absence of any BrdU+/TH+ cells in the substantia nigra in our studies suggests at least two possibilities:

(a) that our procedures are not effectively labeling newly generated dopaminergic neurons in the basal ganglia.

(b) that new dopamine neurons are not generated under the conditions of our studies.

Evidence for (a) comes from Zhao et al. (2003), who reported that repeated *i.p.* injections labeled less than one nigral BrdU+/ TH+ cell per mouse, while 22 cells/animal were labeled using long term *i.c.v.* BrdU infusion. (b) The possibility that new dopamine neurons are not generated in the adult basal ganglia under the conditions in our experiments, which is consistent with the findings of Lie et al. (2002) in the rat. While there were progenitor cells in the adult substantia nigra, they did not differentiate into dopaminergic neurons in normal and 6-OHDA lesioned animals. The absence of neurogenesis in the adult basal ganglia is also consistent with the observations from many groups that neurogenesis in adult mammals is limited to granule cells in the hippocampus and the olfactory bulb (for a critical review, see Rakic, 2002).

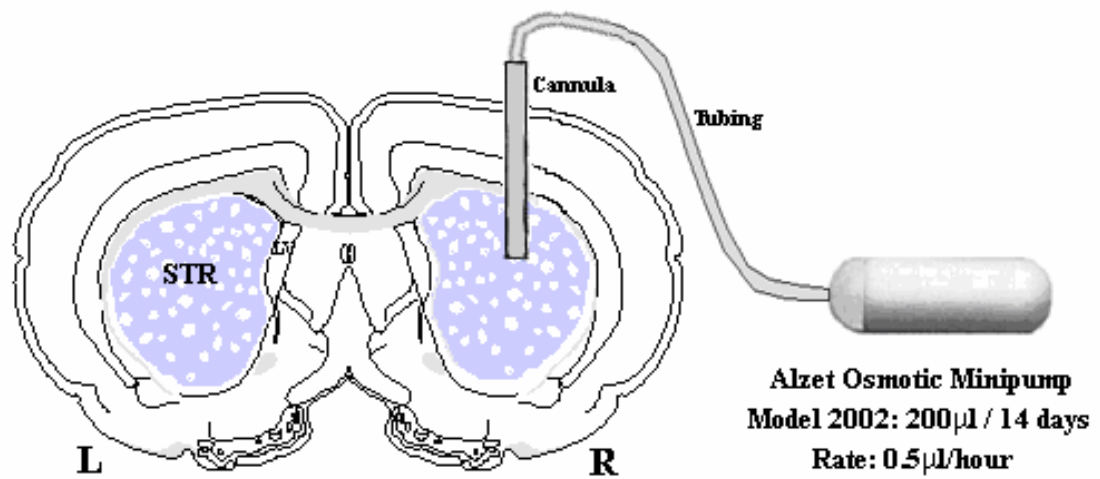
Our next study ([Chapter 3](#)) delivered BrdU through *i.c.v.* infusion and compared the effectiveness of our labeling with the repeated BrdU *i.p.* injections. Later on ([Chapters 4, 5](#)), we tested whether injury, in combination with GDNF treatment, might serve as a positive signal for neurogenesis in the substantia nigra.

Table 2.1 – Antibodies used in study

Antibody to	Host/Type	Dilution	Company	Cell Type Immunostained
BrdU	Rat monoclonal	1:200	Accurate	Stem cells and their progeny; Cells undergoing DNA repair
GABA	Mouse monoclonal	1:50	Chemicon	GABAergic neurons
GAD _{65/67}	Mouse monoclonal	1:200	Chemicon	
GDNF	Goat polyclonal	1:200	R & D Systems	Cells with GDNF labeling
GFAP	Mouse monoclonal	1:1,000	Chemicon	Astrocytes
MAP2	Mouse monoclonal	1:200	Chemicon	Neurons
Nestin (Rat401)	Mouse monoclonal	1:20	DSHB	Neuroepithelial stem cells
NeuN	Mouse monoclonal	1:500	Chemicon	Neurons
NG2	Mouse monoclonal	1:200	Chemicon	Immature Oligodendrocytes
Oligoden-drocyte	Mouse monoclonal	1:5,000	Chemicon	Oligodendrocytess
Ox-42	Mouse monoclonal	1:4,000	BD-Pharmingen	Microglia
TH	Mouse monoclonal	1:1,500	Chemicon	Cells, including dopamine neurons, containing tyrosine hydroxylase
TH	Rabbit polyclonal	1:3,000	Pel-freeze	
β-III tubulin (Tuj-1)	Mouse monoclonal	1:1,000	Covance	Early postmitotic embryonic neurons

Table 2.2 - BrdU+ cell counts in the substantia nigra. Every 6th section through the midbrain in each animal was used for cell counting. The number of BrdU+ cells was counted in the substantia nigra on each side of each animal. The total number of BrdU+ cells in each treatment group was summed in this table. Although altogether 1, 549 cells were observed with BrdU immunoreactivity, no BrdU+/TH+ double labeled cell was observed in each area.

	Right Side		Left Side	
<u>Substantia Nigra</u>	Vehicle	GDNF	Vehicle	GDNF
BrdU+/TH+ neurons	0	0	0	0
BrdU+/TH- cells	310	500	304	435



L: Left; LV: Lateral Ventricle; R: Right; STR: Striatum

Figure 2.1 – Illustration of osmotic pump implantation. The osmotic pump filled with infusate (200 µl GDNF or TGF α or aCSF) was implanted subcutaneously and the infusate was delivered directly into the striatum at a rate of 0.5µl/hour. The concentration of the GDNF was 300µg/200µl, the concentration of TGF α was 100µg/200µl. At the 14th day of infusion, the animal was anesthetized and placed back in the stereotaxic device. A small incision was made on the back skin and the pump was replaced by a new one full of the same infusate (200µl).

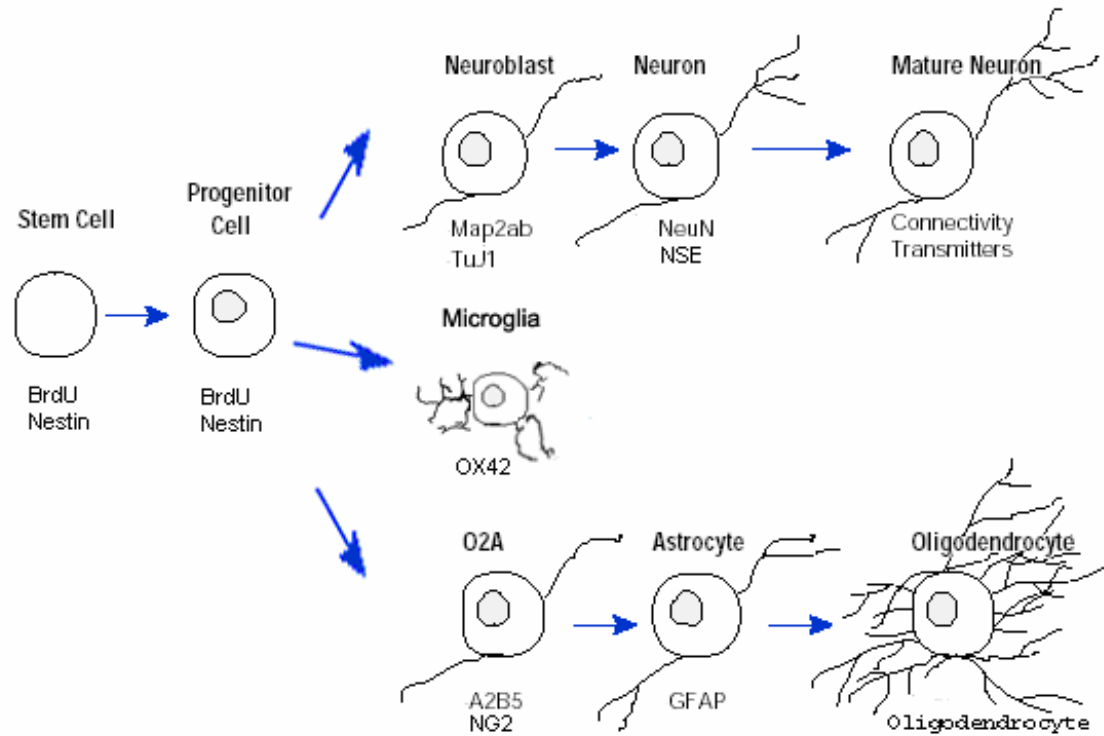


Figure 2.2 – Stem cells in culture express a series of markers as they differentiate and commit to either neuronal or glial lineages.
Figure modified from Palmer et al. 1997.

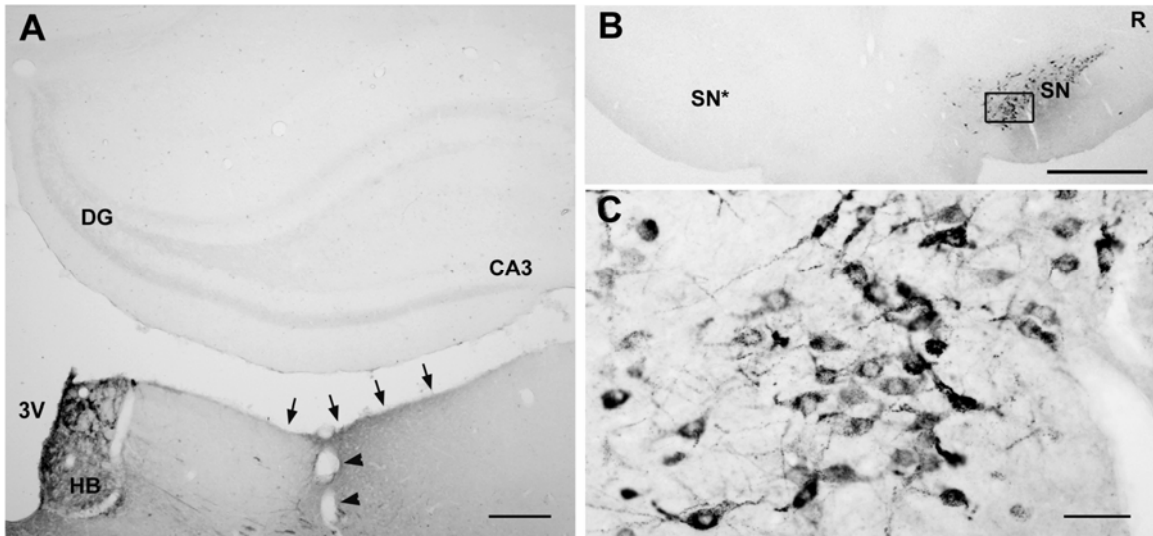


Figure 2.3 – GDNF labeling. A. GDNF immunostaining along the ventricular wall (arrows), indicating that the trophic factor had entered the cerebrospinal fluid (CSF). Note the intense labeling of the habenula (HB). Faint GDNF immunoreactivity was evident in the hippocampus. A large blood vessel (arrowhead) runs from the thalamus to the ventricular wall. Scale bar = 200 μ m. B. GDNF immunostaining in cells in the substantia nigra (SN) on the right side showed retrograde transport from the striatum. In contrast, retrograde transport was restricted to the side of infusion with none of the cells in the contralateral SN (SN*) displaying immunoreactive GDNF. Scale bar = 1mm. C. A higher power magnification of neurons in the SN staining for GDNF (inset from B). Scale bar = 50 μ m.

3V: third ventricle; DG: dentate gyrus; CA3: field CA3 of hippocampus

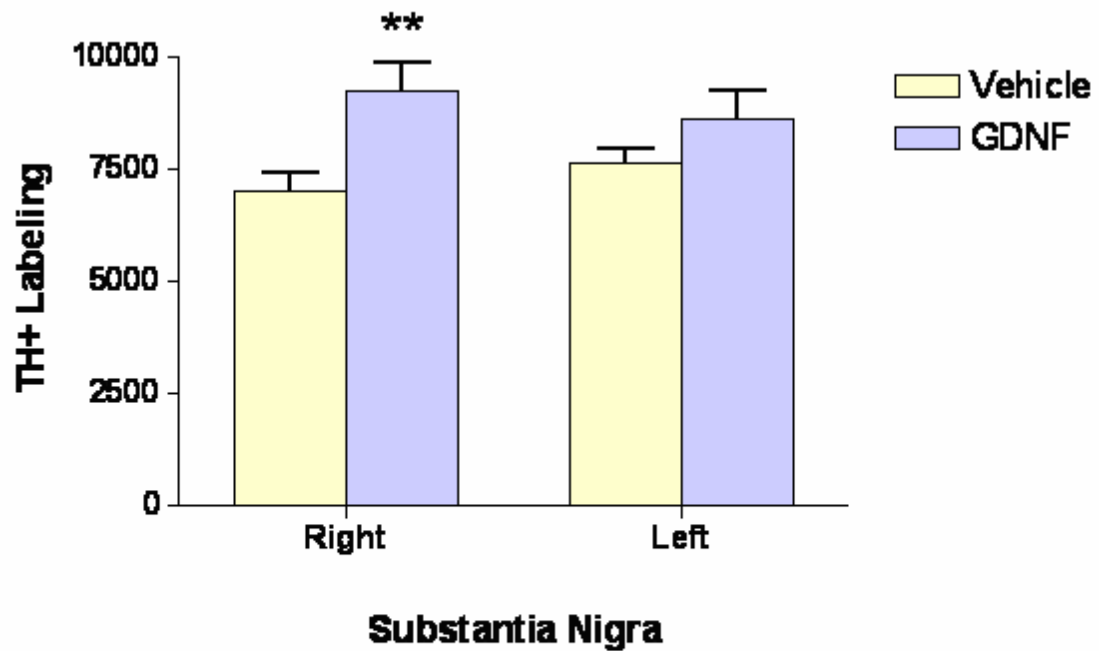
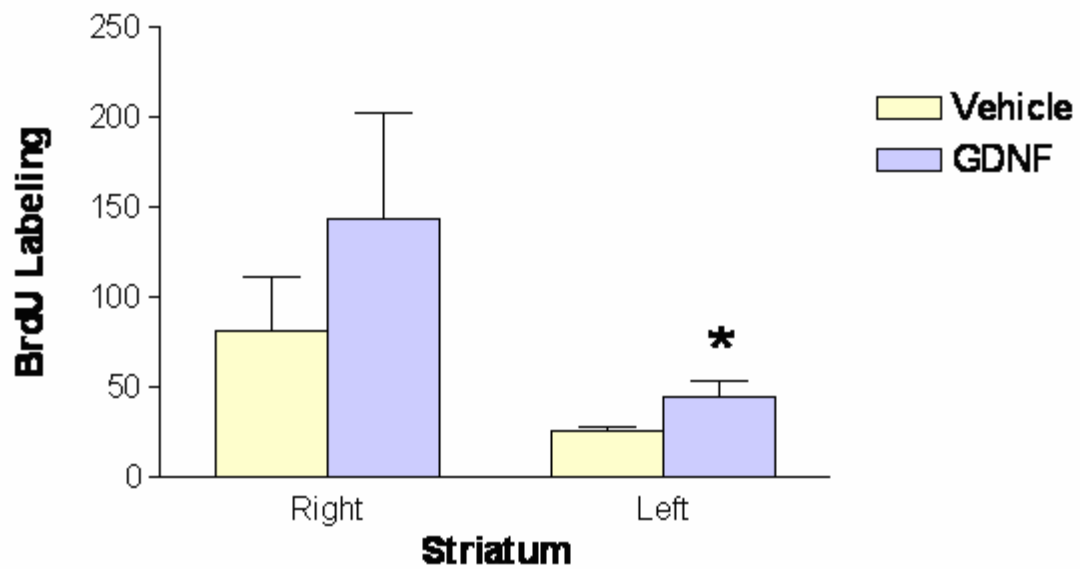


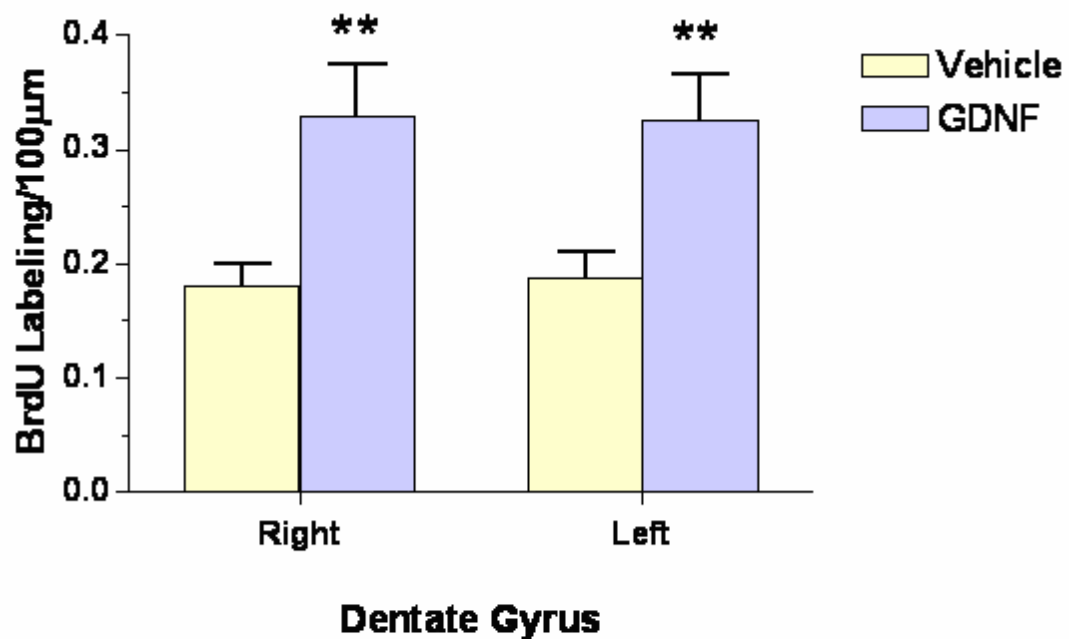
Figure 2.4 – The number of TH labeled cells in the substantia nigra. GDNF-treated animals have higher number of TH immunoreactive cells in both hemispheres than the vehicle recipients. But the difference was significant only on the infusion side. (**: $P < 0.01$)
Right side: $F = 9.248$, $P = 0.008$; Left side: $F = 1.772$, $P = 0.202$.



	Right	Left
Vehicle	81.0 ± 30.4	25.1 ± 2.9
GDNF	142.8 ± 59.5	44.8 ± 8.0

Figure 2.5A – Bioquant cell counting of BrdU labeled cells in the striatum. BrdU labeled cells from 1 in every 6th whole brain sections in the striatum of each animal were screened and counted. There was a significantly higher number of BrdU labeling in the contralateral side with GDNF treatment than with vehicle treatment. (*: $P < 0.05$)

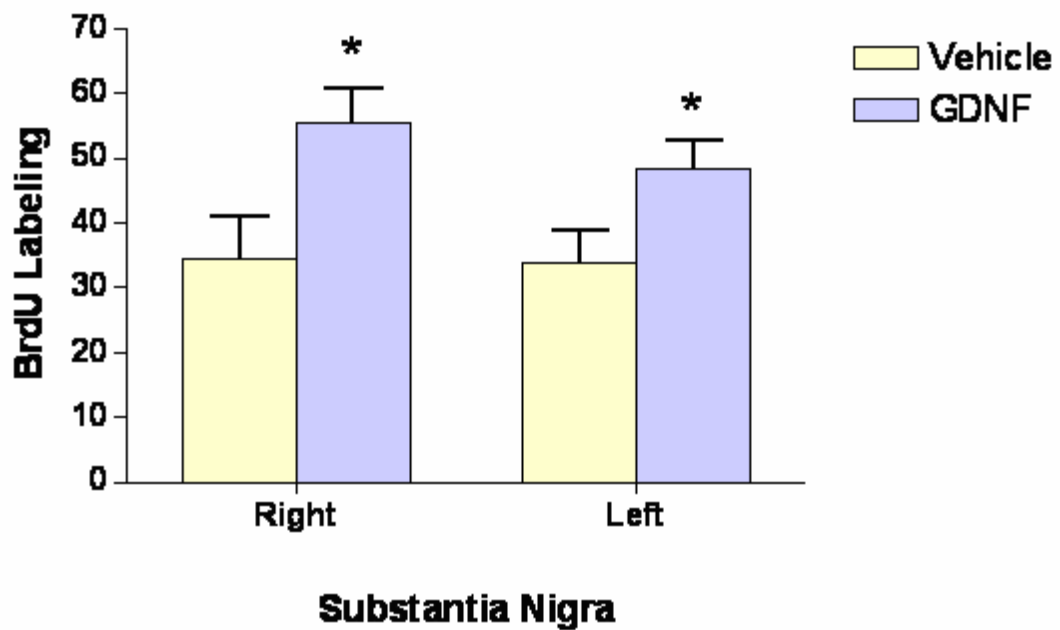
Right side: $F=0.792$, $P=0.388$; Left side: $F=4.849$, $P=0.044$.



	Right	Left
Vehicle	0.18 ± 0.02	0.19 ± 0.02
GDNF	0.33 ± 0.05	0.33 ± 0.04

Figure 2.5B – Bioquant cell counting of BrdU labeled cells in the dentate gyrus. BrdU labeled cells from 1 in every 6th whole brain sections in the dentate gyrus of each animal were screened and counted. The length of each corresponding dentate gyrus was also measured. The average number of BrdU-positive cells in each 100µm-long segment of the dentate gyrus was calculated for each section and averaged in each side of the drug treatment groups, as shown above. There was significantly higher number of BrdU labeled cells in both sides of the dentate gyrus with GDNF treatment than with vehicle treatment. (**: $P < 0.01$)

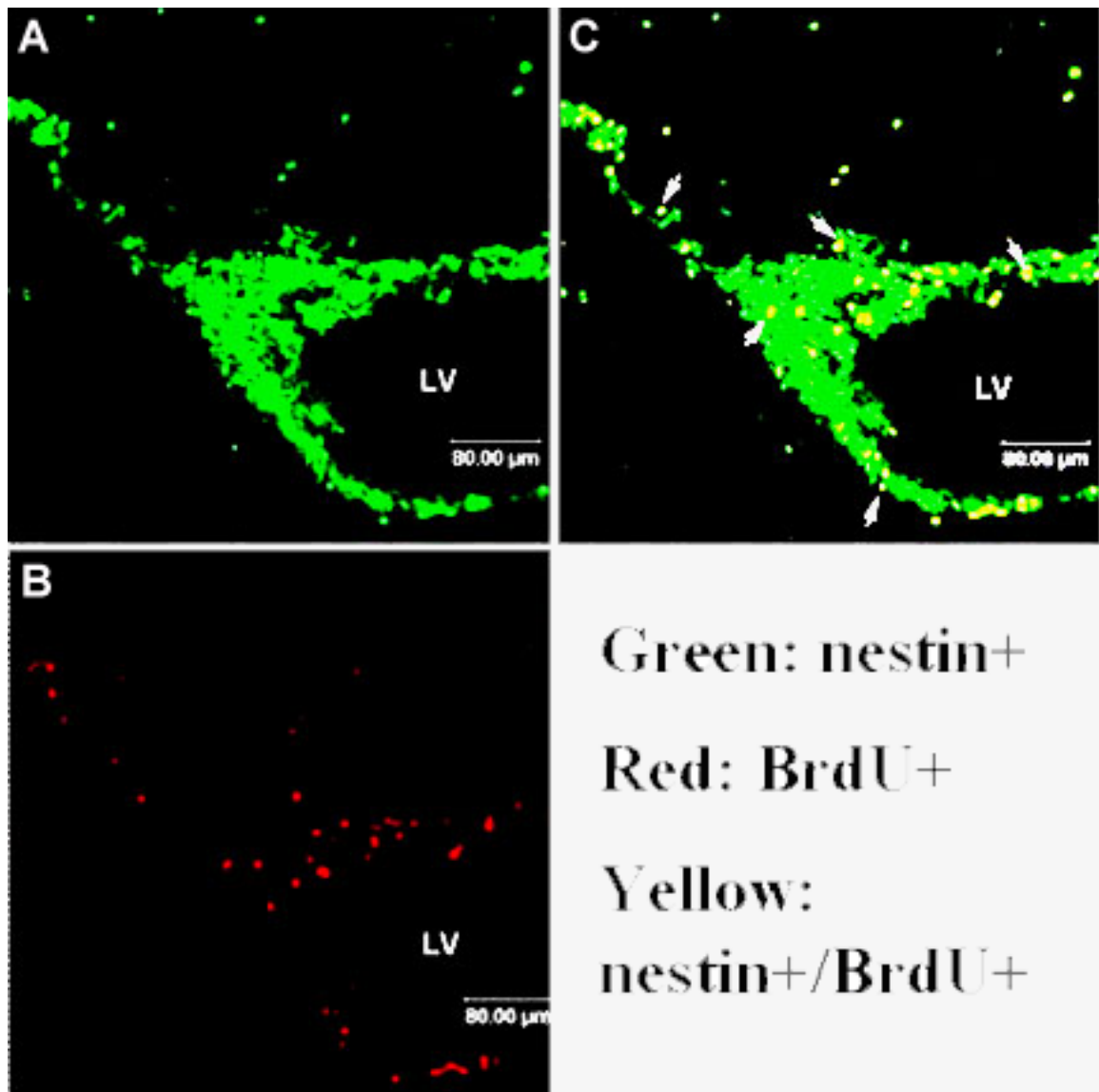
Right side: $F=9.193$, $P=0.008$; Left side: $F=9.141$, $P=0.008$.



	Right	Left
Vehicle	34.4 ± 6.6	33.8 ± 5.1
GDNF	55.6 ± 5.0	48.3 ± 4.4

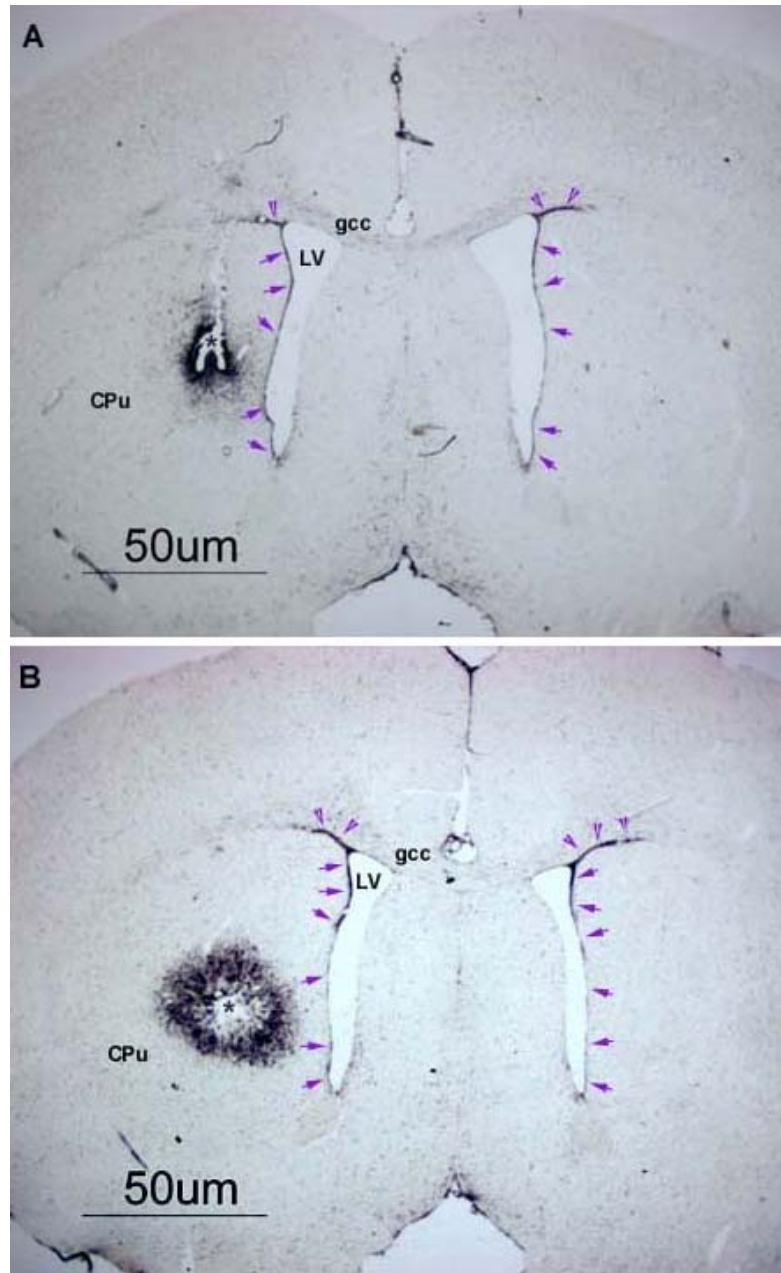
Figure 2.5C – Bioquant cell counting of BrdU labeled cells in the substantia nigra. Number of BrdU labeled cells in the substantia nigra of GDNF-treated animals was significantly higher in both hemispheres than that in the vehicle recipients. (*: $P < 0.05$).

Right side: $F = 6.495$, $P = 0.021$; Left side: $F = 4.744$, $P = 0.045$.



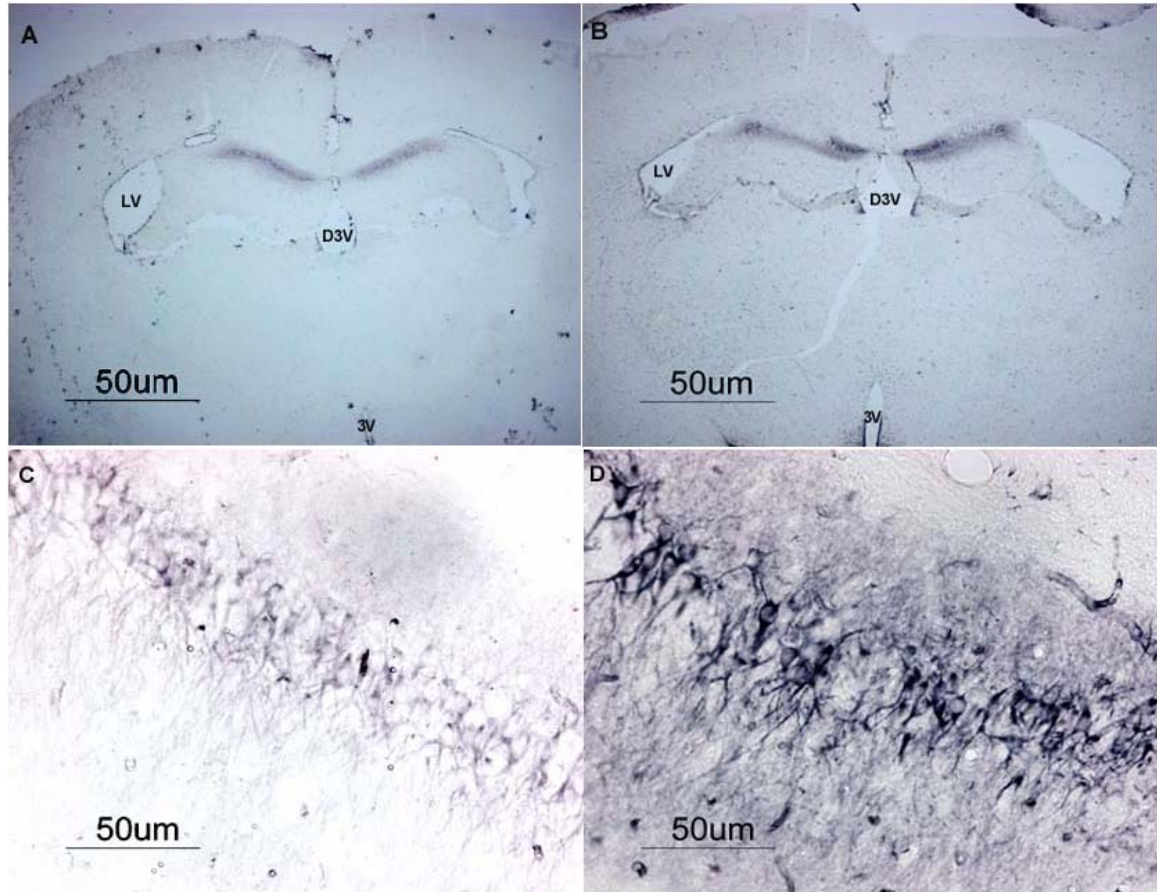
LV: Lateral ventricle

Figure 2.6 – Confocal microscopy was used to confirm the identity of nestin+ cells. Cells in the subventricular zone were stained with nestin (in green, shown in A) and BrdU (in red, shown in B). The two markers were found co-localized (in yellow, shown in C) in many of those cells (arrowheads). This demonstrated a correlation between nestin immunoreactivity and stem cell proliferation.



CPu: Caudate-Putamen; LV: Lateral Ventricle; gcc: Genu Corpus Callosum

Figure 2.7 – Nestin immunoreactivity in the forebrain. Nestin labeling was found in the subventricular zone (arrows) around the lateral ventricle (LV) in both the vehicle-treated animals (A) and GDNF-treated animals (B). The nestin positive fibers also extended (arrowheads) into the striatum in both groups. The intensity of nestin staining was higher in these locations after GDNF treatment. Nestin immunoreactivity was also found around the catheter site (*) in the caudate-putamen (CPu), but most of these cells were microglial cells, not stem cells. Gcc: genu of corpus callosum.



D3V: Third Ventricle; LV: Lateral Ventricle

Figure 2.8 – Nestin immunoreactivity in the hippocampus. Nestin was also expressed in the hippocampus in both the vehicle recipients (A, C) and GDNF recipients (B, D). Again, the nestin immunoreactivity was much stronger in the GDNF treated group, suggesting higher stem cell proliferation in the hippocampus, where neurogenesis has been demonstrated by many investigators to be active in adulthood.

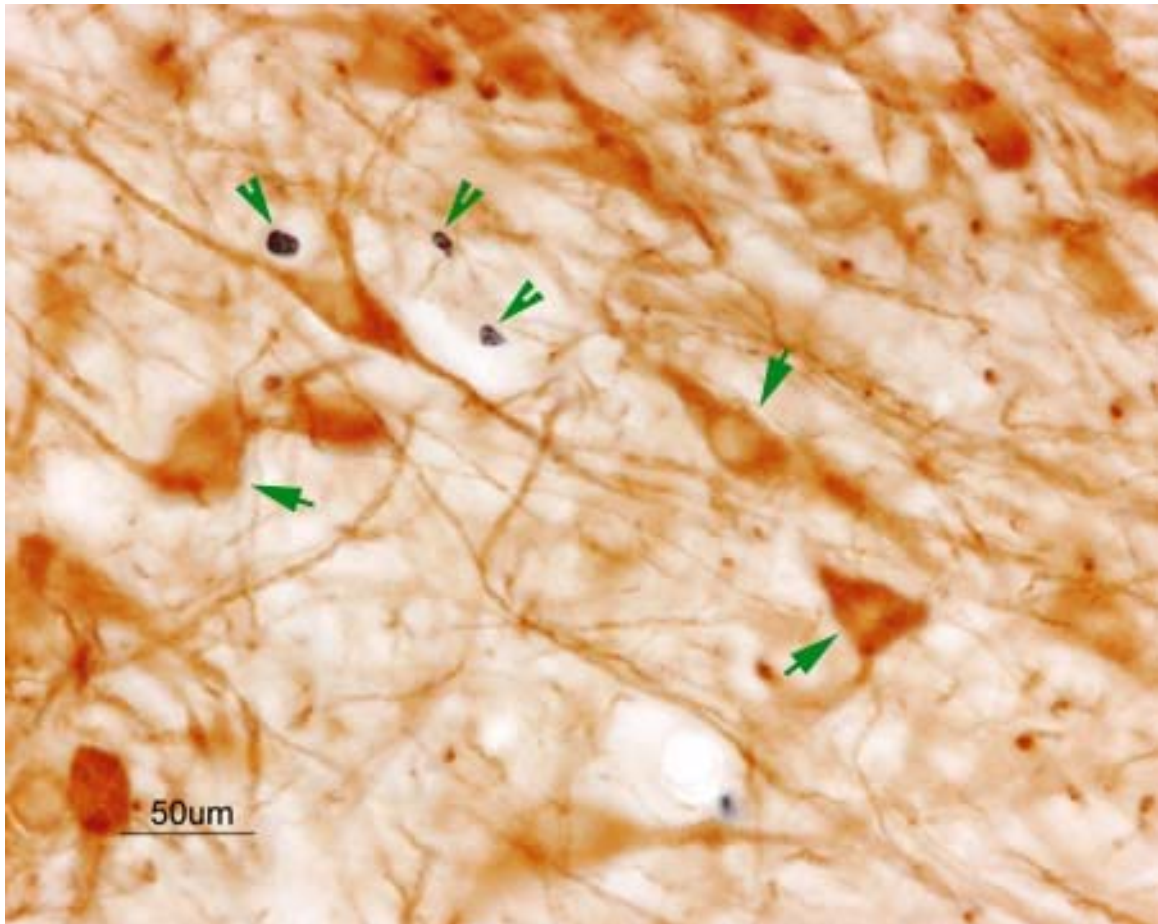


Figure 2.9 – No evidence of neurogenesis in the substantia nigra was observed with traditional double-labeling ICC. ICC for BrdU and TH was conducted in tissue sections containing the substantia nigra to determine the number of cells co-labeling for both markers. TH is a marker for dopaminergic neurons. BrdU positive cells were stained black (arrowheads) and the brown cells were stained with TH (arrows). There were no neurons identified that unequivocally were labeled with both markers.

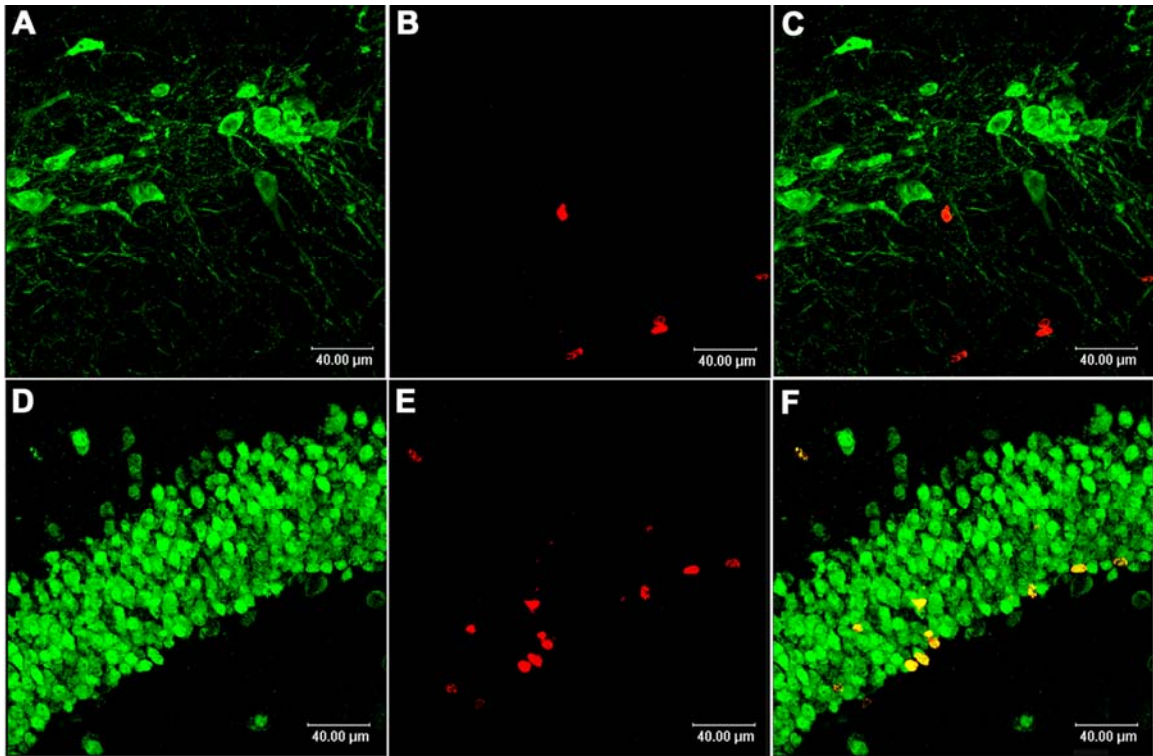


Figure 2.10 – Evidence of neurogenesis in the substantia nigra and hippocampus observed with confocal microscopy. Confocal microscope images illustrating double immunofluorescence staining of BrdU and either TH in the substantia nigra (A-C) or NeuN in the dentate gyrus (D-F) of the BrdU treated rats. A-C. A large number of cells (green) in the substantia nigra were immunostained with TH and a few labeled with BrdU (red). No double labeled cells were found. D-F. In the hippocampal dentate gyrus, neurons were stained with NeuN (green) and a few of them were double labeled (yellow) with BrdU (red).

Chapter Three

Morphology of BrdU Positive Clls in the Striatum, Dentate Gyrus and Substantia Nigra in Normal Animals with Continuous *i.c.v.* Infusion of BrdU

Summary

From our previous study, GDNF has been shown to promote cell genesis in various brain regions including the striatum, hippocampus and substantia nigra. While cells double-labeled for NeuN and BrdU were identified in the dentate gyrus of the hippocampus, no double-labeled neurons were found in the striatum and substantia nigra (Fig. 3.1.). Furthermore, double-label studies using confocal evaluation of BrdU labeled cells for other cell markers (Table 2.1.) were negative. Therefore, the current study was designed to characterize the morphological features of BrdU labeled cells in the striatum, dentate gyrus and substantia nigra at the electron microscopic level. Six rats received direct infusion of 150 mg/ml BrdU in dimethyl sulfoxide (DMSO) into the right lateral ventricle for 28 days, following procedures detailed elsewhere (Zhao et al., 2003). The animals were then euthanized and the substantia nigra region of the brain processed for electron microscopic ICC. Cell features, cell size and nuclear size of the first eight-ten BrdU positive cells identified in the dentate gyrus (Fig. 3.2.) and substantia nigra (Fig. 3.3) region of each animal were evaluated.

Background

BrdU infusion vs. BrdU injection

The route and duration of delivery of BrdU appear to be very important in the report by Zhao et al. (2003). When comparing the two different routes of BrdU delivery for two days, one through continuous *i.c.v.* infusion and the other through repeated *i.p.* injections, they observed a 5-fold higher number of BrdU labeled cells in the dentate gyrus and the substantia nigra with BrdU *i.c.v.* infusion. While the authors observed only one BrdU+/TH+ cell in five mice five weeks after a single *i.p.* injection of BrdU, an average of 22 newly generated dopaminergic neurons were observed in the substantia nigra in two mice treated with BrdU continuous *i.c.v.* infusion for 21 days. Since our study was carried out with BrdU repeated *i.p.* injection similar to theirs, this raised a question of the effectiveness of our technique in identifying stem cells and their progeny. Thus, we tested the existence and extent of neurogenesis with BrdU continuous infusion with similar dosage and duration to the previous report. Most recently, Zhao's methods were repeated and the results challenged by Frielingsdorf et al (2004).

The advantage of 3,3',5,5'-tetramethylbenzidine (TMB) as a chromogen in detecting BrdU labeling

The chromogen TMB is used for pre-embedding ICC to visualize BrdU labeled cells. As a noncarcinogenic analog of benzidine, it is routinely used as the key substrate for enzyme immunoassays involving horseradish peroxidase tagged antibodies. In our experiments, it develops a deep blue color in the presence of peroxidase.

This chromogen has the advantage of being higher performing and more sensitive than the other peroxidase chromogens (Rye et al. 1984).

In this particular study, the antigen BrdU presents inside the nucleus. The BrdU staining with DAB as a chromogen would have very similar appearance as chromatin in the nucleus, thus giving us false positive signals. On the other hand, the BrdU staining with TMB has a crystallized appearance, providing us with a satisfactory way to identify the antigen unambiguously and confidently.

Materials and Methods

Animals

Animals used here have the same characteristics as described in the first study.

BrdU infusion

Altogether ten animals were used for a BrdU infusion study. The animals were anesthetized with chloral hydrate (300mg/kg, *i.p.*), and a 3.5 mm cannula attached to the Alzet osmotic minipump (Durect corporation, Cupertino, CA; model 2004: 200µl/28 days), which was stereotaxically implanted into the right lateral ventricle (coordinates- AP: -0.9mm, ML: -1.4mm) using Bregma as a reference. The pump was implanted subcutaneously and the infusate (200µl BrdU solution) was delivered directly into the lateral ventricle at a rate of 0.25µl/hour. The concentration of the BrdU solution was 150mg/ml in dimethyl sulfoxide (DMSO).

Electron microscopy

Among the animals that received BrdU continuous infusion, six animals were used for electron microscopy. At the end of BrdU infusion, animals were transcardially perfused by a fixative consisting of 4% paraformaldehyde and 0.2-0.5% glutaraldehyde. The brains were collected and postfixed overnight with 4% paraformaldehyde only, and then cut into 50µm sections on a vibratome. First, sections were stained for BrdU using TMB as a chromogen. The process of BrdU labeling was the same as described above with the exception that, instead of visualizing the avidin-biotin-HRP complex with DAB, TMB was used as a chromogen. Reaction in TMB was based on the protocols of Oloucha (1985) and Henry (1985) with minor modifications. Briefly, the sections were removed from the ABC solution and rinsed three times in 0.1M phosphate buffer (pH 6.0). Then the sections were incubated in the TMB solution consisting of 48.75ml of 0.25% molybdic acid in 0.1M phosphate buffer, pH6.0 and 2.5mg TMB in 1.25ml pure EtOH for 20 minutes. 0.5 ml of 0.3% hydrogen peroxide was then added to this solution containing the sections. When labeling was observed, the sections were removed and rinsed in Tris buffer, pH7.4, for one minute. The TMB reaction product was stabilized in a DAB/Cobalt/H₂O₂ reaction solution (consisting of 0.05% DAB, 0.025% cobalt chloride, and 0.001% H₂O₂ in Tris Buffer, pH7.4). As soon as the product turned blue-black, the reaction was stopped by rinsing the sections in 0.1M phosphate buffer, pH7.3. After the sections were rinsed twice in 0.1M phosphate buffer, they were post-fixed in 1% osmium, rinsed, dehydrated and embedded in Spurr's electron microscopy resin. To identify individual cell types with BrdU labeling in the striatum and substantia nigra,

ultrathin (90 nm) sections were cut with a diamond knife, stained with lead citrate and examined under an electron microscope.

Immunofluorescent staining

Four animals were used for an immunofluorescence study. All of the procedures used were the same as described in the earlier study.

Results

No evidence of neurogenesis was observed in the substantia nigra

Selected sections from the substantia nigra were evaluated by double-label confocal microscopy for other cell markers ([Table 2.1.](#)) to determine if any of the BrdU+ cells co-labeled with specific antigens for GABAergic neurons, immature neurons, oligodendroglia, astrocytes or microglia.

Although there is a higher number of BrdU+ cells throughout the brain with BrdU continuous *i.c.v.* infusion as opposed to BrdU repeated *i.p.* injections, no double-labeled cells were found in the substantia nigra ([Fig. 3.1](#)).

The phenotype of BrdU labeled cells in different areas of the brain under the electron microscope

Altogether seventy two BrdU+ cells from the hippocampus and fifty BrdU labeled cells from the substantia nigra were characterized in the six animals receiving continuous intraventricular BrdU infusion for 28 days (Fig. 3.2.).

Of the 72 BrdU labeled cells from the hippocampus observed, 24 were very close to the basal lamina (Fig. 3.2.C); 17 were close to a neuron (Fig. 3.2.A) and 29 appeared to be solitary cells (Fig. 3.2.B). A majority of the cells (89%) possessed rounded nuclei with very little cytoplasm. The average size of the nucleus is $19\mu\text{m}^2$ while the biggest nucleus encountered had an area of about $47\mu\text{m}^2$.

Among the 50 cells from the substantia nigra, eight were small paired cells (couplets, Fig. 3.3. A), 12 were satellite cells to much larger cells (Fig. 3.3. B) and 30 appeared to be solitary cells. Most of the cells (84%) possessed rounded nuclei, with irregular nuclei (Fig. 3.3. C) found in a minority (16%) of the cells. All were small cells consisting of a nucleus surrounded by a thin rim of cytoplasm. The average area of a cell with a rounded nucleus was $18\mu\text{m}^2$, of which the nucleus occupied $16\mu\text{m}^2$. Cells with irregular nuclei were even smaller, with an average total area of $14\mu\text{m}^2$, most of which taken by the nucleus with an area of $12\mu\text{m}^2$. On average, the cytoplasm accounted for around 13% of the cellular area.

Discussion

As opposed to the extensive neurogenesis reported by Zhao et al (2003), our results did not show evidence of newly generated neurons even after continuous BrdU infusion at a dosage very similar to theirs. Our results were in consistence with a recent paper (Frielingsdorf et al. 2004), which failed to replicate Zhao's results. The number of BrdU labeled cells increased prominently throughout the brain, especially in SVZ, after BrdU continuous infusion for 28 days. But most of the cells could not be identified by any of the markers we used here.

We used pre-embedding ICC to label the BrdU positive cells before cutting the brain tissue into ultra-thin sections. Since the BrdU ICC required DNA denaturation as a procedure to expose the nucleus, the morphology of many BrdU positive cells was not always pristine. In most of the cases, the cell membrane was damaged and few organelles were left outside the nucleus when observed under electron microscopy. This made it very difficult to identify the phenotype of these cells based solely on their components under electron microscopy.

Even with the damaged cell membrane, one could tell that the cytoplasm was very limited in the BrdU labeled cells, occupying around 13% of the whole cell area on average. This may explain the difficulty of finding a phenotype-specific marker that could stain BrdU positive cells, especially those antibodies that recognize antigens within the cytoplasm.

A high percentage of the BrdU positive cells we observed were located close to a mature neuron. These satellite cells might contribute to some of the "false positive" co-

localization of BrdU and neuronal markers observed with confocal microscopy by us and other researchers.

One month after the start of osmotic minipump implantation, only uncommitted BrdU positive cells were observed. Whether these cells would continue to be uncommitted, or differentiate into a certain cell lineage is still not known. In our future studies, we would extend the time period after BrdU application to observe the differentiation of these cells.

Several other important points are discussed below:

Limitations of the confocal study

Our results are in contradiction to several other papers showing evidence of constitutive neurogenesis in the striatum and the substantia nigra by confocal microscopic study. This technique, however, has its own limitations. It does not allow for the identification of cell membranes, and it is possible that some of these putative double-labeled cells corresponded to small BrdU-positive cells closely associated to neighboring cells. By combining this technique with the electron microscopic study, we could have a more complete look at the identity of the stem/progenitor cells and their progeny.

Astrocytes might serve as stem/progenitor cells in the CNS

When observed under electron microscope, the BrdU labeled cells showed some characteristics of an astrocyte. They have light cytoplasm containing very few ribosomes and intermediate filament. It has long been speculated since the discovery of glia cells in

the CNS that neurons and glia cells originated from different progenitors. As described below, this may not be the case, and astrocytes may directly give rise to mature neurons.

Our results were consistent with several studies (Doetsch et al. 1999 a, b; Laywell et al. 2000; Seri et al. 2001) showing that stem/progenitor cells may exhibit features of astrocytes and express GFAP, the marker widely accepted as the marker for mature astrocytes. By using transgenic mice expressing GFP under the promoter of nestin, Filippov et al. (2003) demonstrated astrocytic features in nestin-GFP-positive cells. One recent publication (Garcia et al. 2004) used two transgenic strategies to show that GFAP-expressing cells residing in the SVZ and SGZ were predominantly responsible for the constitutive neurogenesis in mice. One separate report showed direct evidence that astrocytes may give rise to new neurons in the adult mammalian hippocampus (Seri et al. 2001).

Whether the BrdU⁺ cells we identified in the striatum, hippocampus, and substantia nigra would further differentiate into mature neurons was unknown at this point. To address this issue, we need to wait for a longer period after the BrdU administration with/without GDNF treatment to evaluate the ultrastructural characteristics or the BrdU labeled cells.

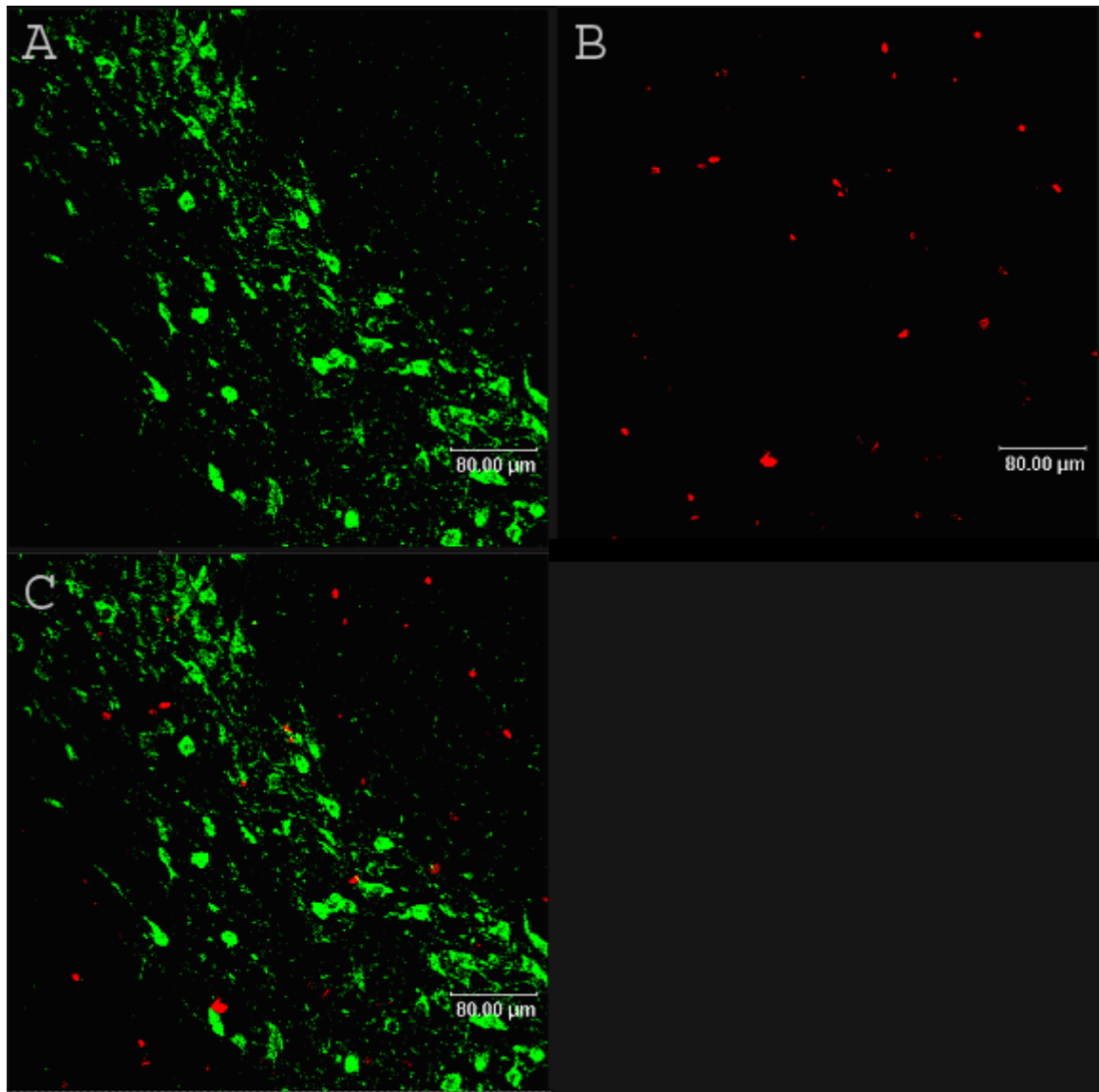
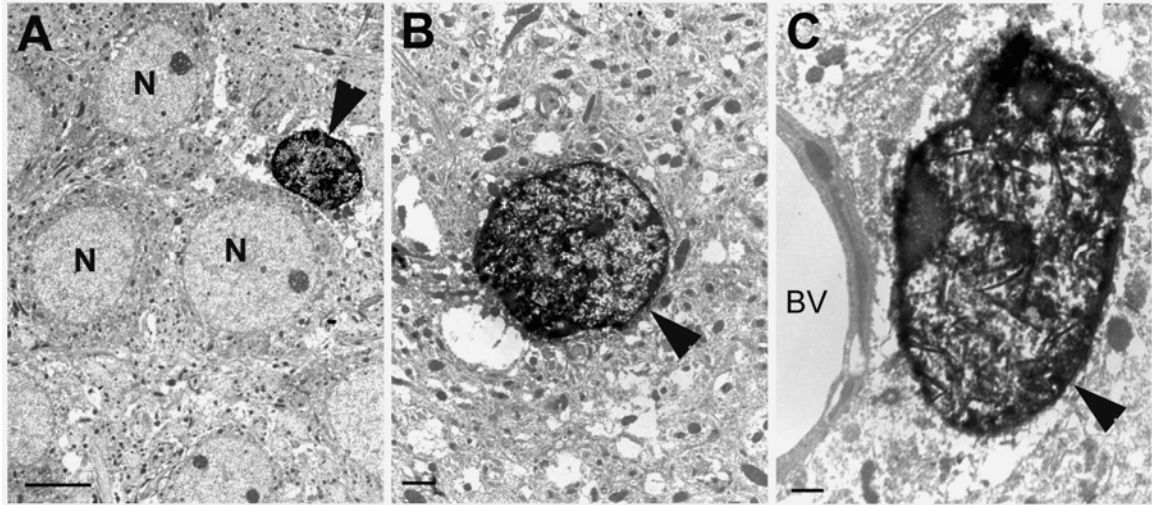


Figure 3.1 – Double-label immunofluorescence staining of BrdU and TH in the substantia nigra of BrdU treated rats. There was extensive BrdU labeling (red) in the substantia nigra from BrdU infusion into the lateral ventricle, but none of the dopaminergic neurons (i.e. TH+ cells, in green) were co-labeled with BrdU (A-C).



N: Neuron; BV: Blood vessel

Figure 3.2 – The morphology of BrdU labeled cells in the dentate gyrus. Immunostaining procedures for BrdU at the electron microscopic level revealed a number of small labeled cells in the hippocampus (A-C). Many of the BrdU labeled cells possessed a rounded nucleus: **A.** Here, a small cell with a BrdU positive nucleus (arrowheads) rests next to granule cells (N) in the dentate gyrus. Scale bar = 500nm. **B.** This is an example of a solitary BrdU-labeled cell in the hippocampus surrounded by *neurites*. Scale bar =1,000nm. **C.** Other BrdU immunopositive cells were located next to blood vessels (BV), with features typical of astrocytes. Scale bar =500nm.

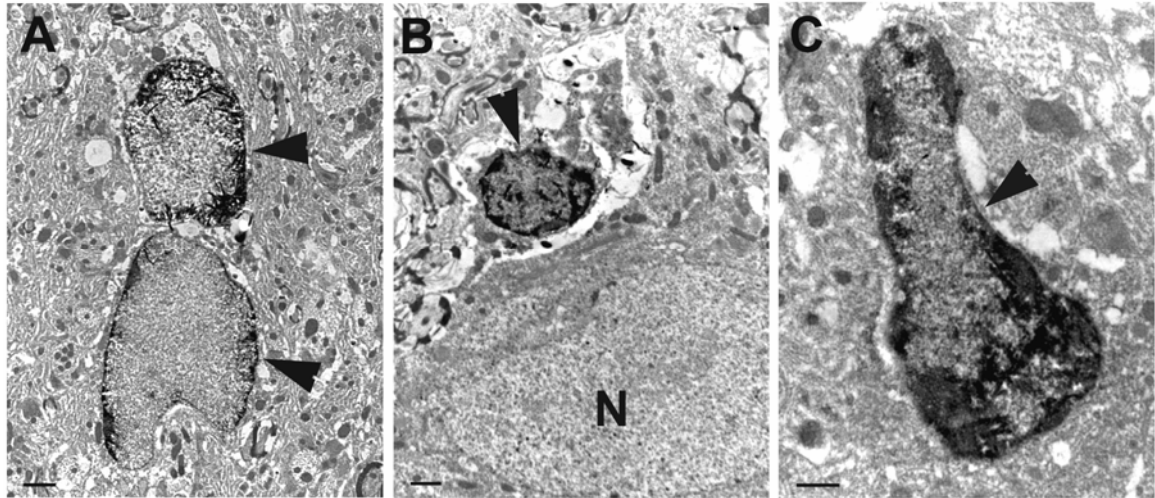


Figure 3.3 – The morphology of BrdU labeled cells in the substantia nigra of animals that received continuous BrdU infusion for 28 days (A-C). **A.** BrdU labeling (arrowheads) in the nucleus of two adjacent cells indicates that they had recently divided. Scale bar =1,000nm. **B.** The typical BrdU-labeled nucleus (arrowheads) in the SN was small, round and intensely labeled. This cell was located next to a neuron (N). Scale bar =1,000nm. **C.** A few labeled cells in the SN showed characteristics of oligodendroglia, with elongated, irregular nuclei containing clumped heterochromatin along the nuclear membrane. Scale bar =500nm.

Chapter Four

The Effects of Intrastriatal Infusion of GDNF One Month after 6-OHDA MFB Lesion on Stem Cell Proliferation and Differentiation in the Striatum, Hippocampus and Substantia Nigra

Summary

While there was no evidence for dopamine neurogenesis in normal young adult rats with GDNF treatment in our first set of experiment, we could not rule out the possibility that GDNF could promote neurogenesis in response to injury. The number of nigral TH+ neurons has been shown to increase with GDNF treatment in several 6-OHDA lesion models (Bowenkamp et al., 1995, Kearns et al., 1997). It has also been reported that injury by itself could induce neurogenesis in neocortex and other brain areas. Therefore, this study was designed to explore the effects of GDNF on neurogenesis in combination with injury. A unilateral 6-OHDA lesion was introduced to produce degeneration of more than 95% of the dopaminergic neurons in the substantia nigra ipsilaterally. This would allow us to identify newly generated cells without interference of the injured cells undergoing DNA repair, which could also incorporate BrdU. One month after the 6-OHDA lesion, the same procedures as in the first study (as described in [Chapter 2](#)) were used in the delivery of GDNF vs. aCSF as vehicle, with the exception that TGF α was not used here. As in the previous study, altogether 600 μ g GDNF were

delivered into each trophic factor recipient during the 28-day period. BrdU was given through repeated *i.p.* injections at the same dosage as in the first study to allow quantitative comparison of BrdU labeling between the two studies. Apomorphine-induced rotation tests were first used to screen the well lesioned animals before GDNF/aCSF treatment and to monitor functional recovery during the drug infusion (Table 4.1.).

We found that GDNF failed to promote significant functional recovery during the period we tested (Fig. 4.1.). Similar to the first study, GDNF treatment significantly increased the number of BrdU labeled cells bilaterally in the dentate gyrus (Fig. 4.2.B) and ipsilaterally in the SNpc (Fig. 4.2.C). We still did not observe any newly generated dopaminergic neurons in the substantia nigra with traditional and fluorescence double-labeling ICC techniques.

Background

Parkinson's disease (PD)

Affecting more than one million Americans alone, PD is a severe neurodegenerative disorder first formally defined by James Parkinson (1817). He noticed the shaking palsy (paralysis agitans) of the patients and described PD as the “saddest of diseases”.

Clinical symptoms of the movement abnormalities include resting tremor, rigidity, bradykinesia (slowness of movement) and postural instability (Hoehn & Yahr, 1998). PD is diagnosed pathologically by the loss of dopaminergic neurons, which are

pigmented, in the SNpc of the midbrain. This results in a depletion of dopamine content in nigral terminal areas in the basal ganglia, especially the putamen (Kish et al., 1988; Jellinger, 2001; Siderowf and Stern, 2003). Another important pathological feature is the presence of neurofilament inclusions (Lewy bodies) in all affected brainstem regions (Lang and Lozano, 1998a; Rascol et al., 2003).

The etiology of PD remains poorly understood. To date, several genes have been found to be related to familial Parkinson's disease including α -synuclein, parkin and ubiquitin carboxy-terminal-hydrolase-L1. However, since only about 5% of PD is inherited, environmental and other factors, such as oxidative stress, mitochondrial dysfunction and lack of neurotrophic support, may play important roles in neuronal degeneration associated with PD (Lindsay et al., 1993; Blum et al., 2001; Steece-Collier et al., 2002; Siderowf and Stern, 2003).

The standard treatment for the disorder is to use a dopamine precursor or dopamine agonists to improve the motor symptoms. So far, L-dopa still remains the most effective drug during the early stages of the disease. However, L-dopa and other dopaminergic medications cannot halt disease progression, and prolonged use of these drugs not only produces unwanted side effects such as daily "on-off" motor fluctuations and dyskinesias (troublesome involuntary movements), but also results in the development of drug resistance in PD patients. Other therapeutic strategies have been developed to complement L-dopa therapy shortcomings. Among them are monoamine oxidase (MAO) B inhibitors, catechol- O-methyltransferase (COMT) inhibitors, anticholinergics and amantadine. Finally, surgical procedures, such as pallidotomy and particularly deep brain stimulation of the globus pallidus pars interna or subthalamic

nucleus are used for advanced PD patients with severe disability (Krack et al., 1999). Although, to a certain degree, these treatments help to relieve some of the symptoms, none of them has been found entirely safe or efficacious (Lindsay et al., 1993; Leonardi and Mytilineou, 1998; Rascol et al., 2003). Taken together, neither pharmacological treatments nor surgical interventions can stop or reverse the degenerative process in the nigrostriatal system.

Although PD is still incurable, progress has been made to treat it with cell replacement therapies (Dunnett et al. 2001). Adrenal chromaffin cells (Olson & Malmfors, 1970), fetal dopamine cells, embryonic stem cells, and special gene-transduced cells (Nakao et al. 2000) have all been used to test for transplantation into certain brain areas, with different extent of success. Despite this, there are a lot of ethical debates and political restrictions imposed upon these procedures, especially in the case of manipulating embryonic stem cells.

Pathological findings from PD patients have shown that around 50%-70% of dopaminergic neurons in the substantia nigra and about 80% of the dopamine content in the putamen are lost by the onset of clinical symptoms (Lindsay et al., 1993; Lang and Lozano, 1998a; Blum et al., 2001). This suggests that the nigrostriatal system has considerable plasticity to compensate for the loss of dopaminergic neurons. Moreover, the spared dopamine cells in the substantia nigra may provide an opportunity for neuroprotective and neurorestorative treatment. The time window between the early stage PD patient (with 50-60% dopamine neurons left) and the advanced PD patient (with only 20-40% dopamine neurons left) can be several years (Bernheimer et al., 1973; Hely et al., 1999). Any treatment that can slow down or stop the dopaminergic cell death during this

period will be very beneficial for PD patients in terms of improving symptoms, changing the quality of life and lengthening patients' life expectancy. One promising therapeutic approach is the use of neurotrophic factors to promote the survival and growth of dopaminergic neurons. The ultimate goal is to slow or halt neuronal degeneration at an early stage and to stimulate compensation and growth in these cells (Moller et al., 1996; Collier and Sortwell, 1999).

GDNF as a promising drug in the treatment of PD

GDNF is the most potent trophic factor for the dopaminergic neurons; it exerts its function by increasing the cell number, cell size, axon sprouting and transmitter uptake (Hudson et al. 1995). The *in vivo* studies also revealed its potent protective effects under several different injury conditions such as axotomy (Beck et al., 1995) or 6-OHDA lesions in rats (Boewenkamp et al., 1995; Kearns and Gash, 1995; Sauer et al., 1995), and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) lesions in mice (Tomac et al., 1995) and rhesus monkeys (Gash et al., 1996). Both 6-OHDA and MPTP lesions have been widely accepted and are used to produce animal models with Parkinsonian features. According to earlier work in our lab (Kearns et al. 1997), administration of GDNF before 6-OHDA lesions can effectively protect and restore TH-immunoresponsive dopaminergic neurons by increasing the number of dopaminergic neurons and the tissue level of dopamine. Similar results were observed in MPTP lesioned rhesus monkeys that received continuous infusion of GDNF into the lateral ventricle or the striatum (Grondin et al. 2002).

Because GDNF and neurturin could rescue dopamine neurons in animal models of Parkinson's disease, hopes have been raised that GDNF family ligands may be new drugs for the treatment of neurodegenerative diseases. Recent clinical trial on PD patients (Gill et al. 2003; Slevin et al. 2004) hold renewed hope for the treatment of this devastating disease.

MFB lesion with 6-OHDA

The structure of 6-OHDA is very similar to that of dopamine. It is a selective neurotoxin (Ungerstedt, 1968; Breese and Traylor, 1970; Uretsky and Iversen, 1970) that can be directly transported into dopaminergic and noradrenergic neurons via high-affinity catecholamine uptake systems. This results in the intraneuronal accumulation of cytotoxic compound, such as H₂O₂ and 6-OHDA quinone. Subsequently, these lead to the degeneration of the terminal axons. When the loss of terminals for a given cell exceeds a certain threshold, the cell bodies may degenerate as well and eventually die (Cohen & Werner, 1994).

Since 6-OHDA cannot pass the blood-brain barrier, it is necessary to administer it directly by stereotaxic injection to target a specific part of the nigrostriatal dopaminergic pathway and produce an animal model of PD. The striatum, the substantia nigra and the MFB are three locations widely used as the target of 6-OHDA delivery.

The MFB consists of the ascending dopaminergic axons collected from both the ventral tegmental area (VTA) and the substantia nigra. It passes to the ventromedial edge of the internal capsule and entered the caudate-putamen by several routes.

When injected into the substantia nigra or MFB, dopaminergic neurons start degeneration within 24 hours and die with no evidence of apoptosis (Jeon et al. 1995).

Injection of 6-OHDA into the MFB not only kills dopaminergic neurons in the substantia nigra and causes extensive loss of cells in the VTA, but also induces degenerative changes of dopamine terminals in the striatum. The 6-OHDA MFB lesion could serve as a good animal model because clinical dysfunctions required very large brain lesions. In the human, mild neurological impairments were correlated with dopamine depletions of 80% in the caudate nucleus and 90% in the putamen, whereas severe deficits were associated with near total dopamine depletions. It has been estimated that the MFB lesion in rats could cause the degeneration of more than 95% of the dopaminergic neurons in the substantia nigra. Extracellular dopamine concentrations can be maintained at adequate levels even after extensive destruction of dopaminergic neurons. However, this compensation would be inadequate to maintain dopaminergic control over striatal cell function after very large lesions, such as 6-OHDA MFB lesion, and eventually would lead to neurological deficits.

Apomorphine-induced rotation test

An apomorphine-induced rotational test was used to assess the extent of neuronal loss and ipsilateral dopamine depletion in the striatum following lesions of the substantia nigra (Mendez and Finn, 1975).

Apomorphine binds to the D2 receptor as a postsynaptic agonist. Injection of apomorphine induces animals to turn in circles vigorously in the direction away from the lesioned side. With a 6-OHDA MFB lesion, more than 95% of dopaminergic neurons had

degenerated. This resulted in an upregulation of D2 receptors on ipsilateral striatum, a phenomenon called denervation supersensitivity. It has been proposed that the receptor denervation supersensitivity is the cause of this behavior. Since we could not get rotation with the limited cell loss seen in 6-OHDA partial lesions, the rotation test could be used to monitor the effectiveness of a 6-OHDA lesion and to identify animals with complete unilateral 6-OHDA lesions (Carman et al., 1991).

In this set of experiments, rats demonstrating a minimum of 300 contralateral turns on average in 50 minutes were considered to be well lesioned. Only complete circling was counted over this time period.

Effects of GDNF on damaged dopaminergic neurons

GDNF has been shown to have potent effects on dopaminergic neurons in response to injury. It could protect against loss of dopaminergic neurons and reduction of striatal dopamine content when administered to rats prior to or shortly after 6-OHDA lesions (Kearns & Gash, 1995; Sauer et al. 1995; Choi-Lundberg et al. 1997; Cass & Manning, 1999). It has also been reported that GDNF protects against dopaminergic neuronal loss and dopamine depletion caused by MPTP and methamphetamine (Tomic et al. 1995; Cass, 1996). When administered after either 6-OHDA lesions or MPTP lesions, GDNF could lead to partial histochemical, neurochemical and functional recovery in rodents and in non-human primates (Hoffer et al. 1994; Bowenkamp et al. 1995; Gash et al. 1996; Lapchak et al. 1997; Cohen et al. 2003).

It has been hypothesized that GDNF specifically affects dopamine neurons in at least three ways.

- (1) Pharmacological effects: it upregulates dopamine neuron activities;
- (2) Neuroprotective effects: dopamine neurons exposed to GDNF are partially protected from some neurotoxins like 6-OHDA;
- (3) Neurorestorative effects: GDNF also promotes the growth and regeneration of dopamine neurons that have been injured, for example, from neurotoxins like 6-OHDA.

There is some evidence that neurogenesis could be a fourth action of GDNF. It refers to an ability of GDNF in promoting the development of new neurons to replace dopamine neurons lost to degenerative processes, such as in PD.

While there was no evidence for dopamine neurogenesis in normal young adult rats in our first experiment, the number of nigral TH⁺ neurons has been shown to increase with GDNF treatment in several 6-OHDA lesion models (Bowenkamp et al., 1995, Kearns et al., 1997). While the interpretation of these data has been that the additional TH⁺ cells represented restored neurons, an alternative interpretation is that they are new neurons.

The neurogenesis of new dopamine neurons, if substantiated, has profound implications for treating Parkinson's disease. It offers the possibility of halting or possibly even reversing the disease process.

Cell genesis in response to injury

The damage of the adult CNS not only breaks the normal communication between healthy neurons, but also initiates a cascade of events leading to neuronal degeneration and death. This includes demyelination of axons, axonal retraction, aberrant axonal sprouting and cell death (Horner & Gage, 2000). In contrast to fish and amphibians, the

adult rodent and mammalian CNS lacks the ability to regenerate new neurons and regrow functional axons after injury. This is not entirely due to the lack of regenerative abilities of the CNS neurons, but also because of the damaged environment that either prevented or failed to support regeneration. In fact, it has been demonstrated for decades that CNS neurons do have regenerative capabilities (Richardson et al. 1980) when placed into the proper environment. Cell survival, axonal growth, re-myelination and synapse formation are four steps required by the regeneration of the CNS.

Transplanting cells from stem cell lines into the damaged brain area has been investigated by a number of groups to determine possible therapeutic applications for neurotraumatic and neurodegenerative diseases. But this approach has its limitations. First of all, there is potential immunological incompatibility between the transplanted stem cells and the host brain. Second, stem cells come from existing cell lines, which may have been maintained *in vitro* for many generations. Chromosomal and genetic abnormalities increase with each generation of cells in culture. Finally, there are ethical issues regarding the use of embryonic stem cells in research; and the sources of stem cells are limited. Thus, an alternate approach, with possibly fewer limitations, would be to activate endogenous adult stem cells and to stimulate their proliferation and differentiation into appropriately functioning neurons.

It has been recently demonstrated that neural stem/progenitor cells migrate to sites of pathological insult such as various types of brain injury and tumors (Arvidsson et al., 2002; Parent et al., 2002; Iwai et al., 2003; Fricker et al., 1999; Aboody et al., 2000; Li et al., 2002). Other studies have shown that injury of the adult brain could promote proliferation of stem/progenitor cells (Parent et al. 1997; 1999; Bengzon et al. 1997; Liu

et al. 1998; Gould & Tanapat, 1997) and reactivate their migration and differentiation (Wang et al., 1998; Leavitt et al., 1999). Limited neurogenesis was also detected after specific lesions in the adult neocortex (Magavi et al., 2000). In some cases, neurogenesis may even support the recovery of function (Nakatomi et al. 2002).

Materials and Methods

MFB lesion

The 6-OHDA MFB lesion model used in this study was generated as described previously (Boewencamp et al. 1995). In the lesion-infusion study, animals were anesthetized with chloral hydrate (Sigma, St. Louis, MO; 300mg/kg, *i.p.*) and received a stereotaxic injection of 6-OHDA into the right MFB (coordinates- AP: -4.4mm, ML: -1.3mm, DV: -8.0mm) using Bregma as a reference. The 6-OHDA solution (9µg/4µl saline containing 0.02% ascorbic acid) was freshly made and injected using a 10µl syringe (Hamilton Company, Reno, NV) at a rate of 0.5µl per minute. The needle was kept in place for an additional five minutes before retraction.

Apomorphine-induced rotation tests

Starting at one week after the MFB lesion, all rats received apomorphine induced rotation tests once a week. The animal received a single subcutaneous injection of apomorphine (0.05mg/kg in saline containing 0.02% ascorbic acid) and was contained in a square box. Their circling behaviors were caught in tapes and only complete circling was counted over 50 minutes. Rats demonstrating a minimum of 300 contralateral turns

on average were considered to be well lesioned and were used for the subsequent infusion experiments. Earlier reports of Hudson et al. (1993) have shown that more than 99% of striatal dopamine content is depleted with similar criteria. The behavioral tests continued for once a week during the drug continuous infusion.

Drug continuous infusion

One month after the 6-OHDA lesion, all rats with good lesions as screened by the apomorphine-induced rotation tests were randomly divided into two groups (n=9). One group received GDNF and another received aCSF. Animals were anesthetized with chloral hydrate (300mg/kg, *i.p.*) and placed in the stereotaxic device. A small hole was drilled in the skull over the right frontal cortex and the 5 mm cannula, which was attached to the Alzet osmotic minipump (Durect corporation, Cupertino, CA; model 2002: 200µl/14days), was stereotaxically implanted into the right caudate-putamen (coordinates- AP: 1.2mm, ML: -2.7mm) using Bregma as a reference. The pump was implanted subcutaneously and the infusate (200µl GDNF or aCSF) was delivered directly into the striatum at a rate of 0.5µl/hour. The concentration of GDNF was 100µg/200 µl. At the 14th day of infusion, the animal was anesthetized and placed back in the stereotaxic device. A small incision was made on the back skin and the pump was replaced by a new one full of the same infusate (200µl).

BrdU injection

The procedures were the same as described in the first study ([Chapter 2](#)).

Tissue collection

At the end (i.e. the 28th day) of drug infusion, the animal was anesthetized with ketamine and sacrificed by transcardial perfusion of 4% paraformaldehyde. The brain was collected and postfixed overnight, and then it was immersed in 30% sucrose for three days before slicing on a freezing microtome. Samples were sectioned at the thickness of 30 μ m and stored in the cryoprotectant at -20°C.

Immunocytochemistry

The same procedures and similar markers were used in this study as the previous studies.

Bioquant

The Bioquant Image Analysis System was used to quantify the newly generated cells in certain brain areas. The areas being investigated included the caudate-putamen and the substantia nigra. The sections used for counting cells in the striatum were labeled with BrdU and counter-stained with neutral red. And the sections used for counting cells in the substantia nigra were double labeled with BrdU and TH. The area (number of pixels) of the regions was quantified. The number of BrdU-positive cells in each region was estimated. On each section, a 300 μ m \times 300 μ m grid was superimposed with a 300 μ m \times 300 μ m counting chamber placed on each intersection. All cells completely within the boundaries of the chamber or crossing the upper or right side of the chamber were counted.

Statistical analysis

The results are expressed as means \pm SEM. Statistical significance in each side between the treatment groups was assessed using two-way analysis of variance (ANOVA) with side as within-subjects repeated factor and treatment as the between-subjects factor. Subsequently, the effect of GDNF treatment was assessed for each side separately by two-tailed Student's t-tests for independent samples. Unequal variance was assumed. The procedures used to compare the BrdU immunoreactivity in the dentate gyrus of the two treatment groups were the same as described in [Chapter 2](#). For apomorphine-induced rotation tests, only full circles each animal turned away from the lesion side in 50 minutes were counted. The number was averaged within each treatment group and presented.

Immunofluorescent staining

Free-floating sections stored in cryoprotectant were used. For fluorescent double-labeling, they were first pre-treated, blocked by 4% normal goat serum, and incubated in anti-BrdU primary antibody (as described above) for 48 hours, and rinsed thoroughly and incubated with Alexa Fluor 568 goat anti-rat antibody (1:500, Molecular Probes, Eugene, OR) for one hour. After being rinsed in PBS, the sections were blocked by 4% normal goat serum again before being incubated in another primary antibody (as listed above) for one to two days. Corresponding to the source of the primary antibody applied, Alexa Fluor 488 secondary antibody was used following complete rinsing in PBS. Fluorescent sections were evaluated using Leica TCS laser scanning confocal microscope systems (Am Friedensplatz, Mannheim, Germany).

Results

BrdU labeling in the striatum, hippocampus and substantia nigra

The number of BrdU labeled cells were quantified by Bioquant software system two months after the 6-OHDA lesion and one month after the start of drug continuous striatal infusion.

In both the dentate gyrus and the substantia nigra, the drug recipients with lesion showed a significantly lower number of stem/progenitor cells than their normal counterpart as described in our first study in [Chapter 2 \(Fig. 2.5.B.-C.\)](#). In the dentate gyrus of lesioned animals, the number of BrdU positive cells in each 100µm-long segment was only about one half of the number in normal animals with the same treatment.

Comparing to vehicle treatment, GDNF did not show significant effects on stem/progenitor cell proliferation in either side of the striatum ([Fig. 4.2.A.](#)). GDNF significantly increased the number of BrdU+ cells in the dentate gyrus bilaterally ([Fig. 4.2.B](#)) and in the ipsilateral substantia nigra ([Fig. 4.2.C](#)). The number of BrdU positive cells in each 100µm-long segment of dentate gyrus in GDNF recipients was increased by ~45%, compared to vehicle controls. The increase was greater in the ipsilateral substantia nigra, where the number of BrdU+ cells was ~167% larger in the substantia nigra of the GDNF recipients.

No significant functional recovery was observed under continuous GDNF treatment in the striatum

As shown in [Fig 4.1](#), although there appeared to be a slight improvement during the first week of infusion in animals treated with GDNF, the recovery did not last for long, and the difference between the two treatment groups diminished after three weeks of drug infusion.

Because of the high variation in each group, overall the recipients of GDNF infusion into the striatum did not show significant functional recovery compared to vehicle recipients at any time point.

No neurogenesis in the substantia nigra was observed under GDNF treatment

We used both the light microscopic double-labeling and confocal immunofluorescent double-labeling techniques to evaluate the existence of neurogenesis. With the exception of BrdU+/Nestin+ cells in the SVZ and BrdU+/NeuN+ cells in the dentate gyrus, no double-labeled cells were found in our survey in any of the treatment groups. An additional search for BrdU+/TH+ cells was conducted using light microscopic double-labeling when counting every BrdU+ cell in every 6th section through the rostral caudal extent of the substantia nigra. While 779 BrdU+ cells were counted in the 18 rats (controls and GDNF-treated) used in this study, none were TH+ ([Table 4.2](#)).

Discussion

One month after the injury, the number of stem/progenitor cells in the striatum, the dentate gyrus of the hippocampus, and the substantia nigra were evaluated with cell counting of BrdU+ cells. When comparing to our first study with drug treatment to normal rat brain, we found fewer BrdU labeled cells in the dentate gyrus and the substantia nigra in drug recipients with 6-OHDA lesions. This result is in accordance with the findings of Hoglinger et al (2004) who showed that there were fewer precursor cells in the SVZ and dentate gyrus in mice treated with MPTP and in PD patients as compared to the control group. This may be due to the loss of dopaminergic control over the SVZ and the SGZ.

GDNF continuous infusion increased the proliferation of stem/progenitor cells in the dentate gyrus and the substantia nigra comparing with aCSF treatment.

Neurogenesis in the dentate gyrus was confirmed by confocal microscopy with BrdU and NeuN. With GDNF treatment, there was a significant increase of BrdU positive cells in both sides of the dentate gyrus with or without injury, suggesting that GDNF might increase neurogenesis in the hippocampus and play an important role in learning and memory. As mentioned before, it would be very important to test whether this kind of stem cell proliferation and differentiation could be linked to the improvement in behavioral tests on cognition.

GDNF did not render further differentiation of stem/progenitor cells into functional neurons in the substantia nigra, though there was a significantly higher number of BrdU+ cells in the ipsilateral side under GDNF treatment.

GDNF did not provide functional recovery to rats one month after the 6-OHDA lesion.

Since the ipsilateral substantia nigra was severely impaired and neuronal projections into the striatum had been lost by one month after 6-OHDA injection into the MFB, we decided to test another experimental approach, one in which we delivered GDNF directly into the substantia nigra before a 6-OHDA lesion ([Chapter 5](#)). The same procedure has been reported (Kearns et al. 1997) to show potent protective effects of GDNF on dopaminergic neurons as measured by improved behavior, neurochemistry and quantitative morphology.

The MFB lesion model

The complete unilateral 6-OHDA-lesion model has its own drawbacks (Lee et al., 1996). First it is the “sudden and massive toxic insult,” resulting in an instant loss of dopamine neurons rather than a gradually progressive neuronal loss. Second, there is a difficulty in administering the proper dosage when a submaximal lesion is desired. However, because our interest here is the function of the newly differentiated dopaminergic neurons in the striatum, it is necessary to eliminate endogenous dopaminergic neurons. The apomorphine-induced behavioral test was used on the animals after the 6-OHDA injections to screen for fully developed lesions. Only those animals that have complete lesions were used for further study.

The lack of functional recovery with GDNF treatment one month after MFB lesion

As seen in [Fig. 4.1.](#), the apomorphine-induced rotation test did not show functional recovery of the lesioned animals with GDNF treatment when compared with the aCSF recipients.

This is the first study by our group in a decade in which intracerebral GDNF treatment has failed to promote behavioral improvements in animals with nigral dopaminergic lesions (Gash et al. 1996 and Grondin et al. 2002).

In this study, we gave a nearly complete lesion of dopaminergic neurons through the MFB, and waited for a month before starting the continuous GDNF infusion. In contrast to similar studies by others (Bowenkamp et al., 1995; Winkler et al., 1996), there was less than 1% of the normal complement of nigral TH+ neurons remaining in the lesioned (data not shown), GDNF treated substantia nigra and MFB was disrupted. Thus, it was hard for GDNF to be retrogradely transported back to the substantia nigra and exert its function.

It is still possible that a population of cells may be responding to the GDNF, but a proper location and timing for GDNF treatment is essential for its function.

Effects of GDNF on stem/progenitor cell proliferation

GDNF treatment significantly increased the number of stem/progenitor cells labeled with BrdU bilaterally in the dentate gyrus of the hippocampus. Since there has been evidence of dopaminergic control over SGZ precursor cell proliferation (Hoglinger et al. 2004), GDNF might be able to exert its function on dopaminergic fibers that form functional connections with the precursor cells.

Besides the hippocampus, there was also a significantly higher number of BrdU+ cells in the ipsilateral substantia nigra in GDNF recipients than in animals receiving aCSF.

The lack of neurogenesis in the substantia nigra with GDNF treatment in response to injury

Since the previous study of BrdU continuous infusion has shown that our procedure is effective in labeling newly generated dopaminergic neurons in the basal ganglia, there remains to be two possibilities in explaining the absence of any BrdU+/TH+ cells in our studies:

- (a) that new dopamine neurons are not generated under the conditions of our studies.
- (b) that our procedures are killing newly generated dopamine neurons.

The apomorphine-induced rotation data ([Figure 4.1.](#)) provided some support that BrdU incorporation may be too detrimental to newly generated dopamine neurons.

Thus, we felt that another set of experiments using milder lesions was needed to determine if dopamine neurogenesis does significantly contribute to GDNF-induced structural and functional recovery of the nigrostriatal dopaminergic system.

Table 4.1 – Procedures undertaken for GDNF/vehicle treatment. Each animal received a unilateral 6-OHDA MFB lesion, and was screened for good lesion before pump implantation. Animals were randomly assigned into two groups, nine animals in each group that received either GDNF or aCSF as vehicle. Behavioral tests were used to test their motor function during the 28 days of drug infusion.

Group No.	Day 1	Day 21, 28	Day 30-57		Day 36, 43, 50	Day 58
			Day 30-34	Day 30-57		
1	6-OHDA Complete Lesion	Rotation Testings	BrdU <i>i.p.</i> injections	GDNF (n=9)	Rotation Testings	Perfusion
2				aCSF (n=9)		

Table 4.2 - BrdU+ cell counts in the substantia nigra. Every 6th section through the midbrain in each animal was used for cell counting. The total number of BrdU+ cells on each side of each treatment group was summed in this table. Although altogether 779 cells were observed with BrdU immunoreactivity, no BrdU+/TH+ double labeled cell was observed in each area.

	Right Side		Left Side	
<u>Substantia Nigra</u>	Vehicle	GDNF	Vehicle	GDNF
BrdU+/TH+ neurons	0	0	0	0
BrdU+/TH- cells	96	257	158	268

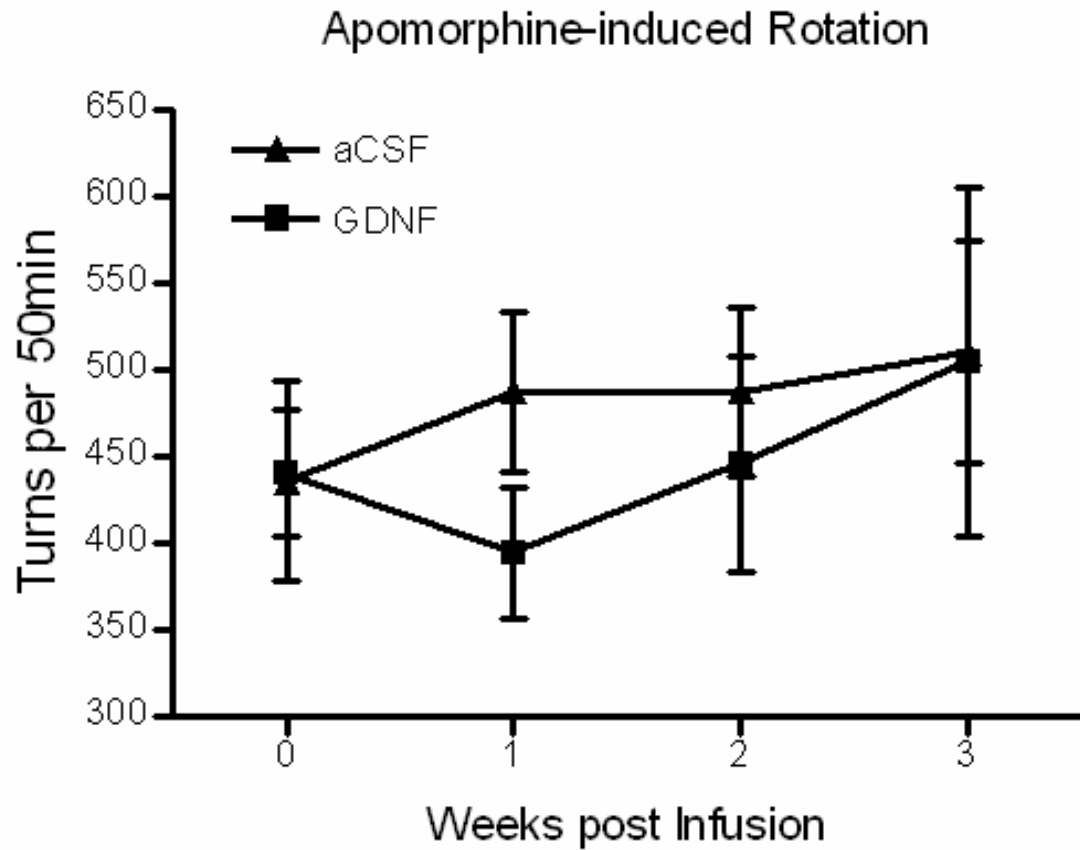
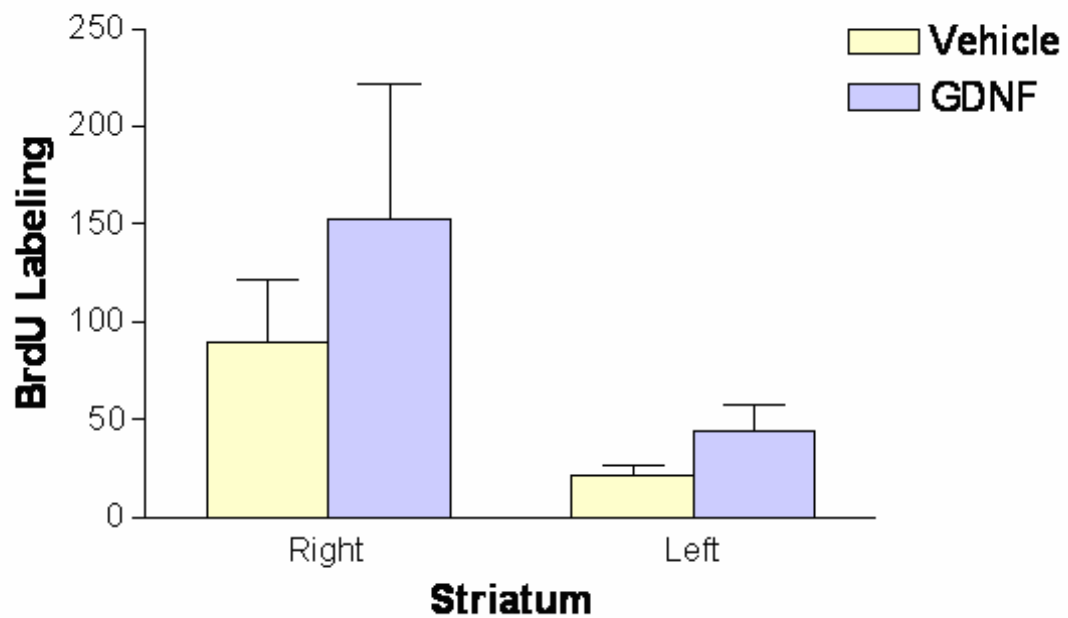


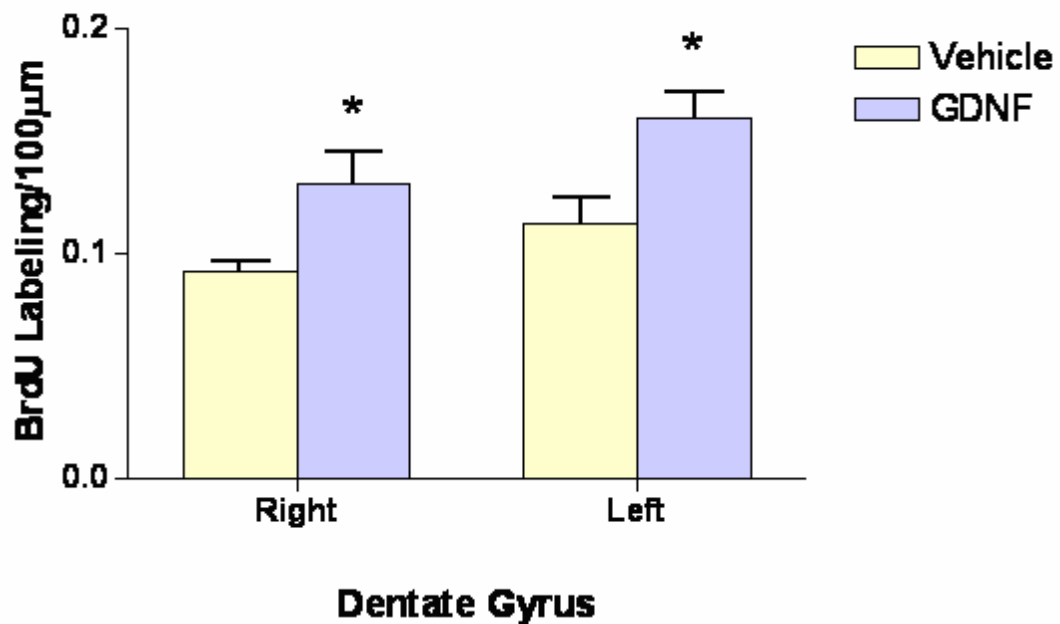
Figure 4.1 – Rotation behavior tests induced by apomorphine administration after either vehicle or GDNF treatment. The graph suggests a slight improvement during the first week of infusion in GDNF recipients, but overall, there is no significant difference between two groups.



	Right	Left
Vehicle	90.0 ± 31.8	22.3 ± 4.6
GDNF	152.3 ± 69.8	44.4 ± 13.7

Figure 4.2A – Bioquant cell counting of BrdU labeled cells in the striatum. BrdU labeled cells from 1 in every 6th whole brain sections in the striatum of each animal were screened and counted. There was no significant difference in BrdU labeling on both sides between the two treatment groups.

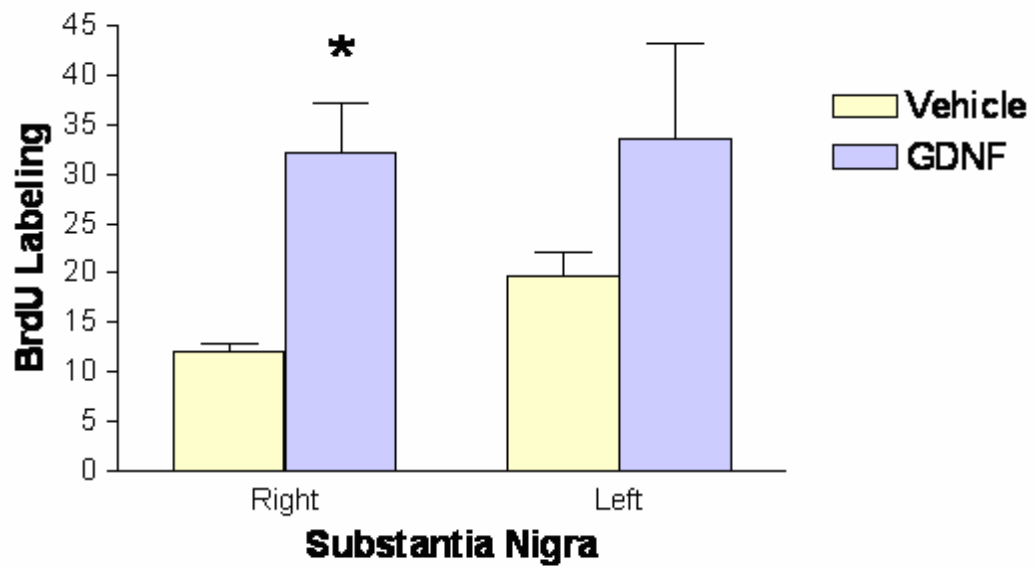
Right side: $F=0.721$, $P=0.411$; Left side: $F=2.646$, $P=0.128$.



	Right	Left
Vehicle	0.092 ± 0.005	0.113 ± 0.012
GDNF	0.131 ± 0.014	0.160 ± 0.011

Figure 4.2B – Bioquant cell counting of BrdU labeled cells in the dentate gyrus. BrdU labeled cells from 1 in every 6th whole brain sections in the dentate gyrus of each animal were screened and counted. The length of each corresponding dentate gyrus was also measured. The average number of BrdU-positive cells in each 100µm-long segment of the dentate gyrus was calculated for each section and averaged in each side of the drug treatment groups, as shown above. There was significantly higher number of BrdU labeled cells in both sides of the dentate gyrus with GDNF treatment than with vehicle treatment. (*: P<0.05)

Right side: F=8.387, P=0.011; Left side: F=7.998, P=0.013.



	Right	Left
Vehicle	12.0 ± 0.8	19.8 ± 2.4
GDNF	32.1 ± 5.0	33.5 ± 9.6

Figure 4.2.C – Bioquant cell counting of BrdU labeled cells in the substantia nigra. Number of BrdU labeled cells in the SN of GDNF-treated animals was significantly higher in the ipsilateral hemisphere than that in the vehicle recipients. (*: $P < 0.05$)

Right side: $F=13.873$, $P=0.002$; Left side: $F=1.703$, $P=0.213$.

Chapter Five

The Effects of a Single Intranigral Injection of GDNF Six Hours before 6-OHDA Partial Lesion on Stem Cell Proliferation and Differentiation in the Striatum and Substantia Nigra

Summary

In this study, we followed the procedures reported by Kearns et al. (1997) to deliver GDNF six hours before a 6-OHDA intranigral (partial) lesion. Consistent with their report, we found improvement of dopamine, 3,4-dihydroxy phenylacetic acid (DOPAC) and homovanillic acid (HVA) tissue levels in both the substantia nigra and the striatum (Fig. 5.1.A-F). Despite this, we still did not observe any newly generated dopaminergic neurons in the substantia nigra. Thus, we conclude that under the experimental protocols we employed, GDNF could not induce neurogenesis in the substantia nigra with or without injury.

Background

The 6-OHDA partial Lesion

As mentioned above, parkinsonian symptoms could be induced by giving animals a neurotoxin 6-OHDA. The toxin results in brain pathology that mimics what is seen in human PD patients. A single stereotaxic injection of 6-OHDA in the SNpc leads to

degeneration of less than 70% dopaminergic neurons, less severe than the MFB lesion. Thus, this produces an animal model with a partial lesion.

GDNF protective effects on dopaminergic neurons

Kearns et al. (1997) found that intranigral injection of GDNF provided protection against a 6-OHDA partial lesion. Young adult male adult Fisher 344 rats were used in the study. GDNF (10µg/2µl) was injected into the SNpc six hours before an injection of 6-OHDA (8µg/2µl) in the same site. ICC staining for TH and HPLC for dopamine and DOPAC were used to observe the morphological and neurochemical effects of GDNF. The results showed that GDNF significantly increased the survival of dopaminergic neurons in the substantia nigra, and the GDNF recipients displayed significantly higher level of dopamine and DOPAC in both the striatum and the substantia nigra three weeks after the lesion than lesioned controls. Interestingly, in the substantia nigra of GDNF pre-treated animals, the number of TH-immunoreactive cells in the lesioned side was consistently higher than the number in the nonlesioned contralateral side. The HPLC results revealed dopamine levels in the substantia nigra of GDNF pre-treated animals that tended to be higher on the lesioned side than on the contralateral side (the difference was not statistically significant).

High performance liquid chromatography (HPLC) on dopaminergic system

The restoration of the striatal dopamine level might be critical to ensure the recovery of dopaminergic function. Dopamine may be inactivated by MAO to release CO₂ and form DOPAC. DOPAC may be further metabolized by COMT into HVA.

Alternatively, if dopamine is acted upon first by COMT, it is transformed into 3-methoxytyramine (3-MT), which may become HVA in the presence of MAO. Both pathways end at HVA, but each has a different intermediate product.

In the long term, dopamine is eventually inactivated by the degradative enzymes MAO and COMT. MAO is predominantly intracellular and COMT extraneuronal. Therefore, tissue levels of DOPAC and HVA will provide an indication of the intracellular and extraneuronal dopamine tissue levels, respectively.

Materials and Methods

Animals and anesthetization

Animals used here have the same characteristics as described in the first study. Nine animals were used in each treatment group.

GDNF and 6-OHDA injections

The animals were randomly divided for two groups. One group received GDNF, and the other group received citrate as vehicle solution. Six hours after the drug pre-treatment, all the animals received a single intranigral 6-OHDA injection.

All surgeries were performed using sterile instruments and aseptic conditions such as disinfections with isopropyl alcohol. The scalp of the anesthetized animal was shaved and the animal placed in the stereotaxic device. The skull was exposed with a small incision and a small hole was drilled in the skull over the posterior cortex for stereotaxic injection of drugs. Either GDNF or citrate was delivered directly over the right SNpc

using the coordinates (AP: -5.4 mm; ML: -2.2 mm; DL: -8.5 mm from skull) through a Hamilton syringe (26 gauge blunt tapered needle). Bregma line was used as a reference for stereotaxic injection. Altogether 2µl 10mM Citrate/120mM NaCl buffer (pH 5.5) or 2 µl GDNF was delivered at a rate of 0.2 µl/minute. The concentration of GDNF solution was 5µg/µl. The needle was left in place for an additional five minutes following the injection and then slowly withdrawn. The incision was closed with wound clips. The animal was kept warm on a heating pad until awaking, and then placed back into the cage with food and water. The animal was closely monitored for signs of distress from surgery and anesthesia.

After six hours, the animal was re-anesthetized by chloral hydrate and the incision was opened. A total of 8µg/2µl 6-OHDA was injected by a Hamilton syringe through the same hole mentioned above with the same stereotaxic coordination. Other procedures of injection were the same as described above. After injection, the incision was closed with wound clips. The rat was returned to the cage after awaking and checked daily within the first week after surgery.

BrdU injection

The procedures were the same as described in the first study ([Chapter 2](#)).

Tissue collection for ICC

One month after the drug treatment and 6-OHDA partial lesion, the animals were anesthetized with Ketamine and sacrificed by transcardial perfusion of 4% paraformaldehyde. The brain was collected and postfixed overnight, and then it was

immersed in 30% sucrose for three days before slicing on a freezing microtome. Samples were sectioned at the thickness of 30 μ m and stored in the cryoprotectant at -20°C.

ICC and immunofluorescent study

The same procedures and similar markers were used here as in the previous studies. The confocal microscope procedures were used to identify BrdU+/TH+ double labeled cells on one twenty-fourth of total brain sections in each animal of both treatment groups.

Tissue collection for HPLC

Animals used for HPLC study were sacrificed by decapitation while under CO₂ anesthesia and the brains were quickly removed. By using an ice-chilled brain mold (Rodent Brain Matrix, ASI Instruments, Warren, MI), a 2-mm coronal slab of brain was cut; the striatum was dissected from each half of the slab separately. Similarly, the substantia nigra was dissected from each side of a 2 mm-thick coronal slab through the midbrain, respectively. The tissue samples were placed in preweighed vials, weighed, and frozen on dry ice. Samples were stored at -70 °C until assayed for dopamine and its metabolites by HPLC (Hall et al. 1989) using electrochemical detection.

HPLC

Tissue levels of dopamine and DOPAC from each side of the striatum and substantia nigra were determined by HPLC analysis with electrochemical detection (Hall et al. 1989). Peaks were identified by retention times of standards. Peak heights were

used to quantify the recovery of internal standards and the amounts of monoamines and metabolites. Results were expressed as ng/g wet weight of tissue.

Statistical analysis

The results are expressed as means \pm SEM. Levels of dopamine and DOPAC were expressed as nanograms per gram of wet weight of tissue and averaged within each treatment groups as presented. Statistical significance in each side between the treatment groups was assessed using two-way analysis of variance (ANOVA) with side as within-subjects repeated factor and treatment as the between-subjects factor. Subsequently, the effect of GDNF treatment was assessed for each side separately by one-tailed Student's t-tests for independent samples. Unequal variance was assumed.

Results

HPLC study confirmed protective effects of GDNF on dopaminergic neurons

In GDNF recipients, dopamine levels in ipsilateral substantia nigra of the lesioned animals were significantly higher than that in the citrate recipients ([Fig 5.1.A](#)).

Levels of the dopamine metabolites were also measured. The tissue levels of DOPAC ([Fig. 5.1.C](#)) and HVA ([Fig 5.1.E](#)) in the ipsilateral striatum was significantly higher in the GDNF treated group than that in the citrate treated group.

No neurogenesis in the substantia nigra was observed under GDNF treatment

No BrdU+/TH+ or BrdU+/DAT+ cells were observed in the substantia nigra with the double-labeling immunofluorescence study.

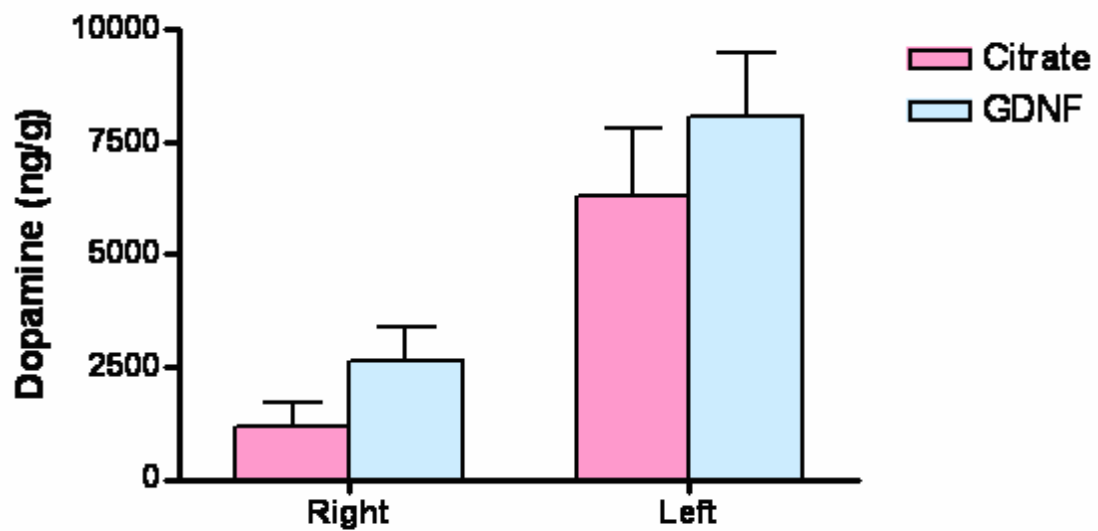
Discussion

GDNF is a potent trophic factor on dopaminergic neurons with protective and restorative effects. It has been reported repeatedly that the number and size of dopaminergic neurons increased in the substantia nigra under GDNF treatment in response to injury (Beck et al. 1995; Tomac et al. 1995; Gash et al. 1996). Specifically in rats with 6-OHDA lesions, GDNF has been reported to have strong neuroprotective and neurorestorative effects on the dopaminergic system (Boewenkamp et al. 1995; Kearns and Gash, 1995; Sauer et al. 1995). This study followed almost exactly the procedures used previously by Kearns et al (1997) in their neuroprotective study. In their report, there were significant increases of the dopamine and DOPAC tissue levels in 6-OHDA lesioned animals with GDNF treatment. Thus, we used the one-tailed t-test in comparing the dopamine and DOPAC tissue level in the striatum and the substantia nigra in this study. Our HPLC results were comparable to the neurochemistry results reported in that publication. The dopaminergic neurons were completely protected by GDNF according to the paper. Based on our observation, the GDNF recipients in our experiments seem to have more TH+ cells in the substantia nigra compared to the vehicle treated group. Since the paper by Kearns et al. (1997) reported significantly increased number of TH+ cells with GDNF treatment and our focus was on the existence of neurogenesis, we did not quantify the number of TH labeled cells to show how significant the difference was.

Since we were more interested to see whether GDNF could induce the local micro-environment to allow the production of newly generated neurons in the substantia nigra, we did not count the BrdU labeled cells in the dentate gyrus and the basal ganglia as we did in the previous studies.

Although there was evidence of increased neurogenesis in response to several brain insults including seizure and ischemia (Magavi et al. 2000; Nakatomi et al. 2002), the 6-OHDA lesion did not induce the production of newly generated dopaminergic neurons in the substantia nigra as shown by our confocal study with BrdU and TH antibodies.

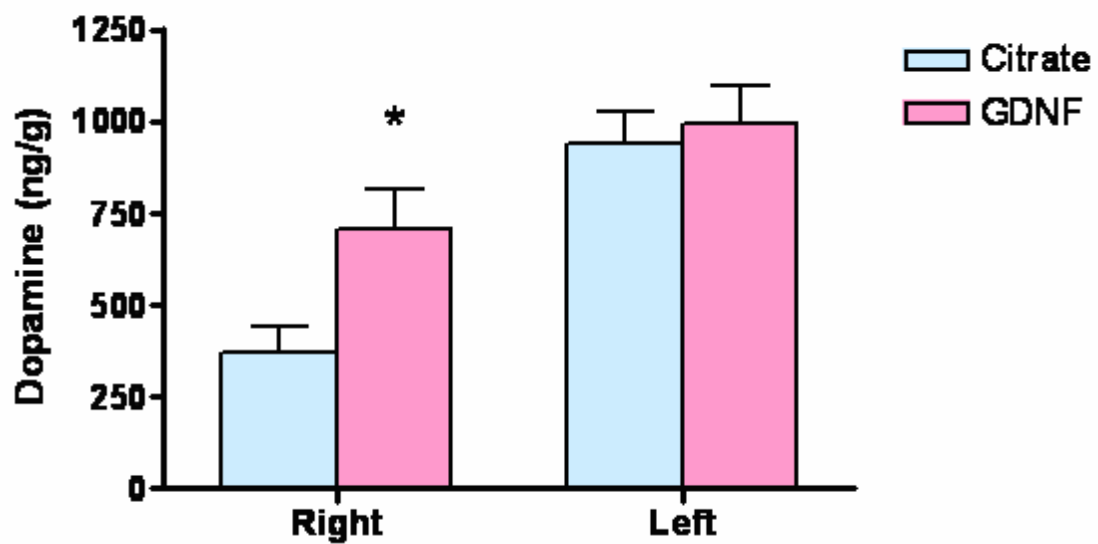
Combined with the results of our earlier study with the 6-OHDA MFB lesion model, we believe that the absence of dopaminergic neurogenesis was neither due to the location and time frame of GDNF delivery, nor related to the extent of the lesion. Despite the existence of stem/progenitor cells locally, the substantia nigra may not offer a suitable environment for the production of new neurons.



	Right	Left
Citrate	1188.2 ± 508.9	6295.5 ± 1504.0
GDNF	2648.3 ± 743.5	8061.5 ± 1389.1

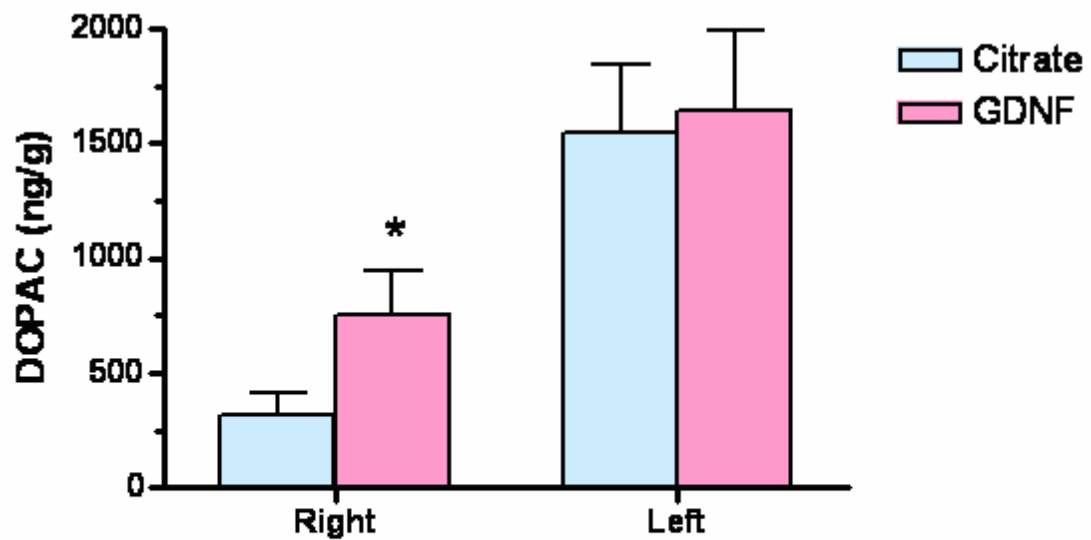
Figure 5.1.A – HPLC results on dopamine tissue level in the striatum. The tissue level of dopamine in the caudate-putamen was compared between the GDNF treated group and the Citrate treated group. Although the GDNF recipients had higher DOPAMINE overflow in both sides of the striatum than the vehicle recipients, the difference was not significant.

Right side: $F=2.744$, $P=0.056$; Left side: $F=0.729$, $P=0.205$.



	Right	Left
GDNF	703.8 ± 109.1	994.0 ± 100.5
Citrate	369.8 ± 70.3	937.3 ± 88.9

Figure 5.1.B – HPLC results on dopamine tissue level in the substantia nigra. The tissue level of dopamine was compared between the two treatment groups in the substantia nigra. The dopamine overflow in the right substantia nigra with GDNF recipients was significantly higher than that of the vehicle recipients. (*: $P < 0.05$)
 Right side: $F = 3.662$, $P = 0.039$; Left side: $F = 0.181$, $P = 0.339$.



	Right	Left
Citrate	321.7 ± 92.7	1552.9 ± 287.5
GDNF	753.9 ± 192.5	1646.6 ± 348.5

Figure 5.1.C – HPLC results on DOPAC tissue level in the striatum. The tissue level of DOPAC was compared between the two treatment groups in the substantia nigra. The DOPAC tissue level was significantly higher in the right striatum with GDNF recipients than that of the vehicle recipients. (*: $P < 0.05$)

Right side: $F=4.457$, $P=0.028$; Left side: $F=0.044$, $P=0.419$.

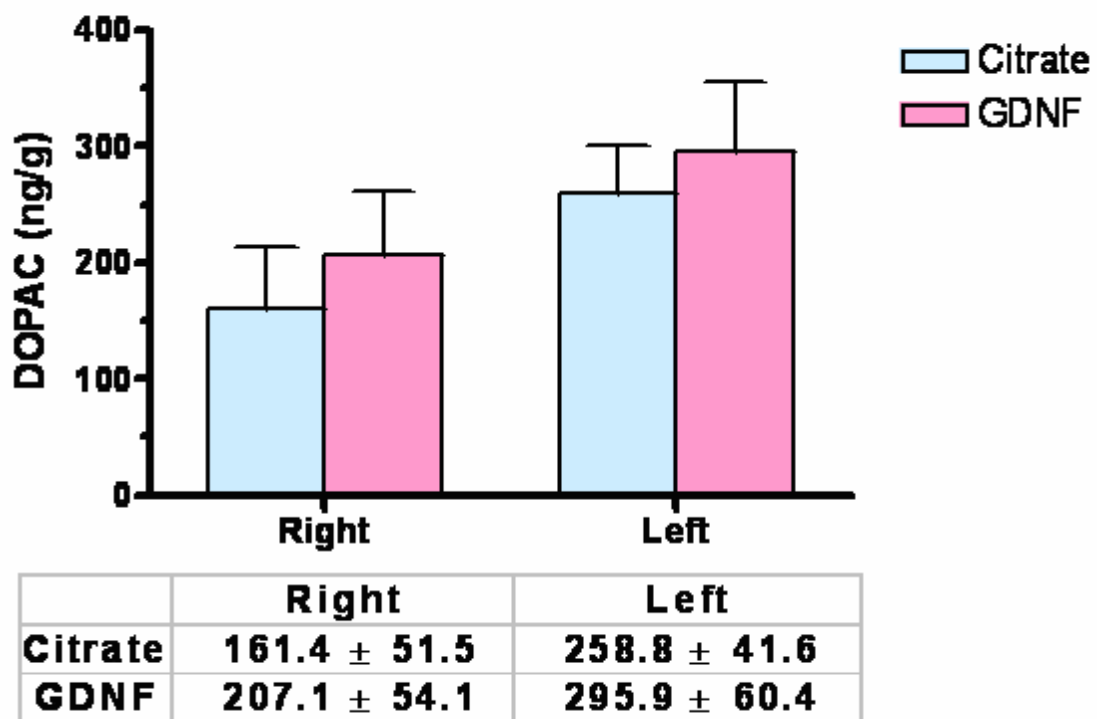
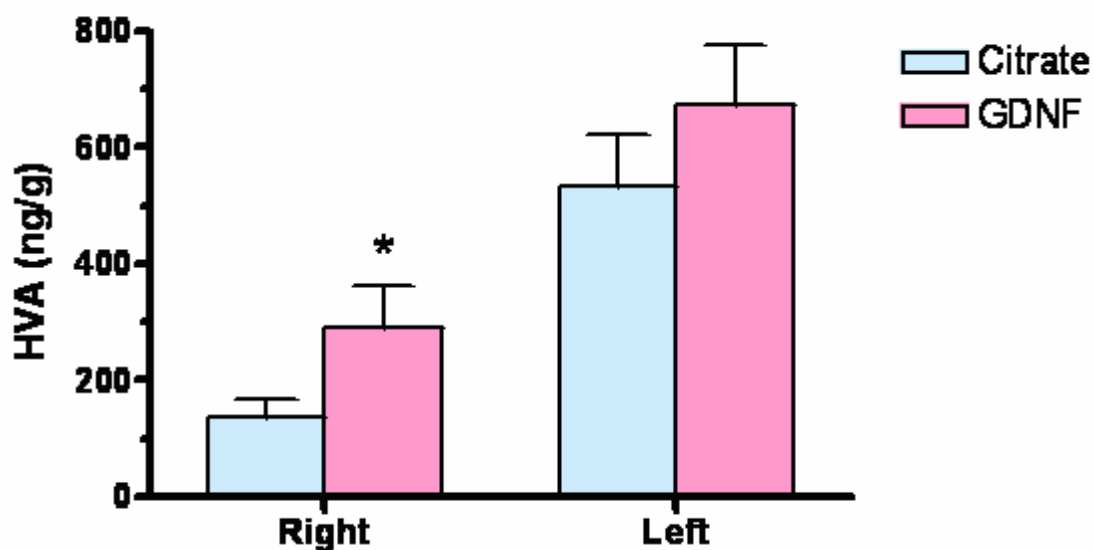


Figure 5.1.D. – HPLC results on DOPAC tissue level in the substantia nigra. The tissue level of DOPAC was compared between the GDNF treated group and the citrated treated group. The difference was not significant in either side of the substantia nigra.

Right side: $F=0.371$, $P=0.277$; Left side: $F=0.267$, $P=0.307$.



	Right	Left
Citrate	135.8 ± 29.8	531.5 ± 89.7
GDNF	290.0 ± 71.8	671.6 ± 102.8

Figure 5.1.E – HPLC results on HVA tissue level in the striatum. The tissue level of HVA was compared between the two treatment groups in the substantia nigra. The HVA tissue level was significantly higher in the right striatum with GDNF recipients than that of the vehicle recipients. (*: $P < 0.05$)

Right side: $F=4.330$, $P=0.029$; Left side: $F=9.141$, $P=0.161$.

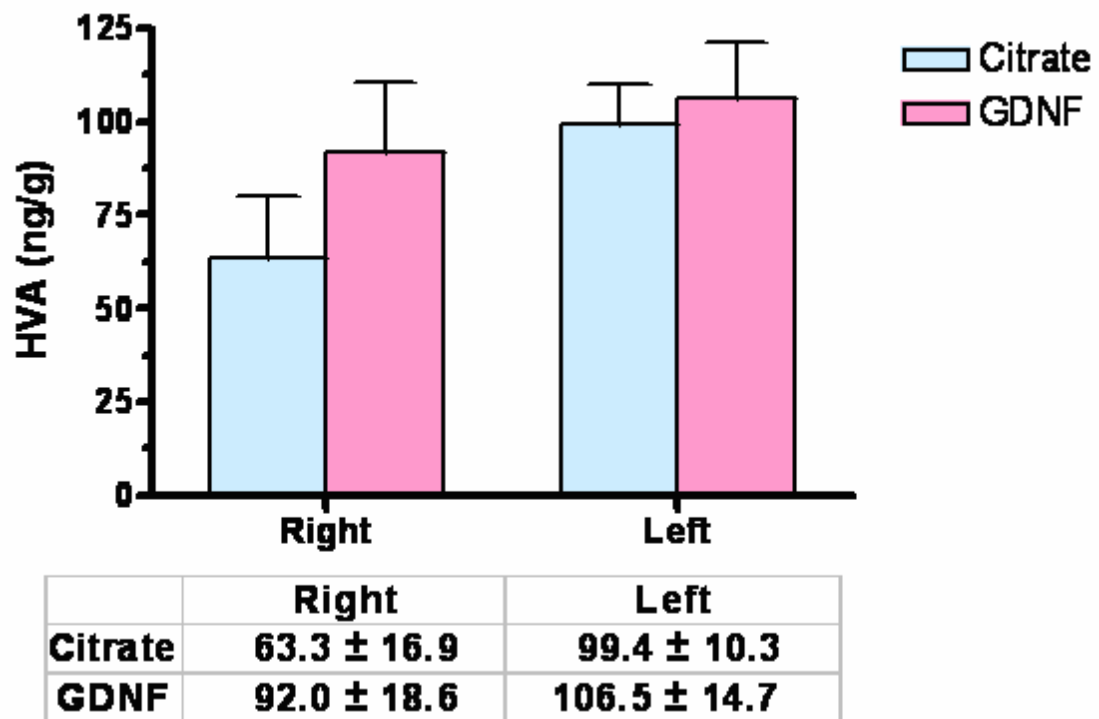


Figure 5.1.F. – HPLC results on HVA tissue level in the substantia nigra. The tissue level of HVA was compared between the GDNF treated group and the citrated treated group. The difference was not significant in either side of the substantia nigra.
 Right side: $F=1.310$, $P=0.137$; Left side: $F=0.162$, $P=0.347$.

Chapter Six

General Discussion and Conclusions

In the past decade, GDNF has been identified as the most potent trophic factor for the dopaminergic neurons in the brain and the motoneurons in the spinal cord. Application of GDNF has increased dopaminergic cell number, cell size, axonal sprouting, and dopamine uptake in the brain of normal, aged and Parkinsonian animals. Besides, it may also exert function in the SVZ and hippocampus, where adult stem/progenitor cells exist and proliferate constitutively.

The primary purpose of this study was to evaluate the general hypothesis that GDNF induces stem/progenitor cells to proliferate and differentiate into neurons in the basal ganglia and the dentate gyrus of the hippocampus. Four specific aims were derived from this overall hypothesis: first, we found that GDNF enhanced stem/progenitor cell proliferation in normal rat brain, but did not produce new dopaminergic neurons. Second, we examined the ultrastructure of the BrdU labeled cells in the striatum, hippocampus and substantia nigra with continuous infusion of BrdU. What we found is that the results of the first study were not due to limitations in the delivery of BrdU. In addition, any BrdU labeled cells that possessed features of differentiated cells showed characteristics of astrocytes. Then, we investigated the effect of continuous GDNF infusion on the 6-OHDA MFB lesioned brain, and found increased BrdU proliferation in the hippocampus and the ipsilateral substantia nigra. There were no newly generated dopaminergic neurons observed under GDNF treatment even in response to the severe injury. Last, we explored

the involvement of neurogenesis in the protective effect of GDNF on the basal ganglia following a well established study. Although dopamine and DOPAC levels were increased with GDNF treatment, still no evidence of neurogenesis was detected in the substantia nigra.

To simplify our experimental design, we used male Sprague-Dawley rats exclusively. Since ovarian hormones such as estrogen have been shown to increase cell genesis in the dentate gyrus (Tanapat et al. 1999; Ormerod and Galea, 2001; Daszuta et al. 2001), it would be interesting to investigate the effect of sex difference on neurogenesis in the future.

The most exciting finding in this project was the potent bilateral effects of GDNF in stem/progenitor cell proliferation in the dentate gyrus of both normal and lesioned animals. This is the first time that GDNF has been linked to increased neurogenesis in the hippocampus. Neurogenesis has been found to correlate very well with the improvement of learning and memory in normal, aged and lesioned animals (Shors et al. 2001; Kemperman et al. 1997, 1998). In our experiments, GDNF administered intrastrially through continuous infusion diffused through the entire ipsilateral hemisphere including the striatum, corpus callosum, cortex and substantia nigra (Fig.2.3). Since *i.c.v.* administration of GDNF improved spatial learning in aged rats as reported by Pellemounter et al (1999), we would be very interested to test whether GDNF treatment in normal and lesioned rats could enhance learning and memory and to further evaluate the involvement of neurogenesis in this mechanism.

While double-labeled BrdU+/NeuN+ cells in the dentate gyrus were observed with confocal microscopy procedures (Fig.2.10.) in normal and lesioned rats, we did not

detect any BrdU labeled cells in the hippocampus that have the characteristics of a mature neuron. Besides, about one fourth of the BrdU labeled cells detected in both the hippocampus and substantia nigra were located very close to a neuron. These cells may contribute to some of the “false positive” double labeling of BrdU and neuronal markers under traditional and confocal microscope. This indicates that confocal microscopy, though a successful technique in detecting colocalization of different cell markers, may be misleading unless all of the three different planes (x, y and z) were scanned on each section and some other techniques including electron microscopy were used as a supplementary.

The nestin-immunoreactivity was more extensive in the SVZ and hippocampus of the GDNF-treated group than in the vehicle recipients, as depicted in [Fig.2.7](#) and [Fig. 2.8](#). These results further confirmed the increase stem/progenitor cell proliferation with GDNF infusion.

In our electron microscopy study, BrdU labeled cells were either undifferentiated or showed characteristics of astrocytes. It has been reported (Doetsch et al. 1999 a, b; Filippov et al. 2003; Laywell et al. 2000; Seri et al. 2001) that astrocytes might serve as stem/progenitor cells that could give rise to mature neurons (Garcia et al. 2004). Our results were consistent with these observations and offered direct evidence that stem/progenitor cells might have ultrastructural characteristics of mature astrocytes.

GDNF showed potent effects on stem/progenitor cell proliferation in the basal ganglia. It was difficult to compare the number of BrdU labeled cells in the caudate-putamen between the two treatment groups without the interference of the local immune response toward catheter implantation; however, there was a significantly higher number

of BrdU positive cells in the contralateral striatum of normal animals with the GDNF treatment. The BrdU immunoreactivity was significantly higher in the substantia nigra of GDNF recipients under normal conditions or after severe 6-OHDA MFB lesions. Based on our electron microscopy study on normal animals, BrdU labeled cells were not undergoing apoptosis. Here we reported, for the first time, that GDNF promotes stem/progenitor cell proliferation in the basal ganglia. Since GDNF is a potent trophic factor on dopaminergic neurons, stem cell biology might be involved in the mechanisms by which GDNF exerts its functions.

Normal animals, upon receiving GDNF, have a significantly higher number of dopaminergic neurons labeled with TH in the ipsilateral substantia nigra. Since we did not detect any newly generated neurons in the substantia nigra under GDNF/vehicle treatment, GDNF might simply increase the TH immunoreactivity instead of neurogenesis. Another explanation was that GDNF helped to promote locally presented dopaminergic precursors to differentiate through enhanced gliogenesis.

Based on previous studies (Rakic, 2002; Lie et al. 2002), the local micro-environment of stem/progenitor cells were critical for their differentiation. As described by Lie et al (2002), endogenous stem/progenitor cells exist in the substantia nigra and have the ability to proliferate into all cell lineages including neurons when isolated and cultured *in vitro*. They had the ability to differentiate into glial cells *in vivo*. Furthermore, these stem/progenitor cells could survive and differentiate into neurons when transplanted back into the hippocampus, where neurogenesis presents constitutively. No neurogenesis was found in the basal ganglia with GDNF treatment under normal conditions or with injury in our studies, suggesting that GDNF by itself may not be able to alter the

microenvironment in the substantia nigra and allow the differentiation of endogenous stem/progenitor cells locally present.

With GDNF intrastriatal treatment one month after 6-OHDA MFB lesions, motor functions were not improved as measured by apomorphine-induced rotation tests. This suggests that the location and time point of drug delivery, as well as the severeness of the lesions, may be critical for GDNF to exert its function. The following study, with GDNF intranigral administration six hours before a partial 6-OHDA lesion, showed neurochemical improvements under GDNF treatment without neurogenesis. This further confirmed that GDNF alone might not be sufficient to induce neurogenesis despite its potent neuroprotective and neurorestorative effects on dopaminergic neurons.

Up until now, there have been two reports on the observation of newly generated dopaminergic neurons either in the striatum of animals with intrastriatal TGF α treatment (Fallon et al. 2002), or in the substantia nigra of normal and MPTP lesioned animals (Zhao et al. 2003). As reported in the second paper, the rate of neurogenesis even doubled after MPTP lesions. Both papers were repeated carefully and independently with minor modifications by us and other groups (Cooper & Isacson, 2004; Frielingsdorf et al. 2004; reviewed by Lindvall et al. 2004). In all cases, the existence of neurogenesis could not be proved. Other groups also could not detect the presence of neurogenesis in the substantia nigra (Lie et al. 2002; Mao et al. 2001) using various experimental design. We suspect that the observation of neurogenesis in the basal ganglia may be “false positive” signals due to the limitation of cell markers (Nowakowski & Hayes, 2000; 2002) or the confocal microscopy as described above.

In each of our experiments, we observed the effect of GDNF only 28 days after the start of its delivery. This time point was chosen because significant structural and functional recovery of the nigrostriatal system has been reported in the lesion models being used by four weeks after GDNF treatment (Bowenkamp et al., 1995; Kearns et al., 1997; Fox et al., 2001). As observed in our second study with BrdU infusion, many of the marker labeled cells seem to be uncommitted progenitors under the electron microscopy. Thus, further experiments are needed to extend the time point and investigate the long term effects of GDNF on neurogenesis.

In conclusion, we are still many steps away from inducing endogenous neurogenesis in the substantia nigra, but the discovery that GDNF treatment induced prominent stem/progenitor cell proliferation gave us a new insight in understanding the mechanism of its function. In addition, the effects of GDNF on neurogenesis in the dentate gyrus and the hippocampus-associated learning and memory need to be further investigated with great promise.

References

- Aboudy K.S. et al. (2000) From the cover: neural stem cells display extensive tropism for pathology in adult brain: evidence from intracranial gliomas. *Proc. Natl. Acad. Sci. USA*. 97: 12846-12851.
- Ai Y., Markesbery W., Zhang Z., Grondin R., Elseberry D., Gerhardt G.A. and Gash D.M. (2003) Intraputamenal infusion in aging rhesus monkeys: distribution and dopaminergic effects. *J. Comp. Neurol.* 461: 250-261.
- Airaksinen M.S. and Saarma M. (2002) The GDNF family: signaling, biological functions and therapeutic value. *Nat. Rev. Neurosci.* 3: 383-394.
- Altman J. (1962) Are neurons formed in the brains of adult mammals? *Science* 135: 1127-1128.
- Altman J. and Das G.D. (1965a) Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. *J. Comp. Neurol.* 124: 319-335.
- Altman J. and Das G.D. (1965b) Post-natal origin of microneurons in the rat brain. *Nature* 207: 953-956.
- Anderson M.F., Aberg M.A., Nilsson M., and Eriksson P.S. (2002) Insulin-like growth factor-I and neurogenesis in the adult mammalian brain. *Brain Res. Dev. Brain Res.* 134(1-2): 115-122.
- Arenas E., Trupp M., Akerud P., and Ibanez C.F. (1995) GDNF prevents degeneration and promotes the phenotype of brain noradrenergic neurons *in vivo*. *Neuron* 15(6), 1465-1473.

- Arvidsson A., Collin T., Kirik D., Kokaia Z., and Lindvall O. (2002) Neuronal replacement from endogenous precursors in the adult brain after stroke. *Nat. Med.* 8: 963-970.
- Baloh, R.H., Tansey, M.G., Lampe, P.A., Fahrner, T.J., Enomoto, H., Simburger, K.S., Leitner, M.L., Araki, T., Johnson, E.M. Jr, and Milbrandt, J. (1998). Artemin, a novel member of the GDNF ligand family, supports peripheral and central neurons and signals through the GFRalpha3-RET receptor complex. *Neuron* 21(6): 1291-1302.
- Barbacid M. (1995) Neurotrophic factors and their receptors. *Curr. Opin. Cell Biol.* 7(2): 148-155.
- Barde Y (1989) Trophic factors and neuronal survival. *Neuron* 2:1525–1534.
- Bayer S.A., Yackel J.W., and Puri P.S. (1982) Neurons in the rat dentate gyrus granular layer substantially increase during juvenile and adult life. *Science* 216: 890–892.
- Beck K.D., Valverde J., Alexi T., Poulsen K., Moffat B., Vandlen R.A., Rosenthal A., and Hefti F. (1995). Mesencephalic dopaminergic neurons protected by GDNF from axotomy-induced degeneration in the adult brain. *Nature* 373,339 -341.
- Bedard A., Cossette M., Levesque M., and Parent A. (2002) Proliferating cells can differentiate into neurons in the striatum of normal adult monkey. *Neurosci. Lett.* 328: 213-216.
- Bengzon J., Kokaia Z., Elmer E., Nanbhashvili A., Kokaia M., and Lindvall O. (1997) Apoptosis and proliferation of dentate gyrus neurons after single and intermittent limbic seizures. *Proc. Natl. Acad. Sci. USA.* 94: 10432-10437.

- Benraiss A., Chmielnicki E., Lerner K., Roh D., and Goldman S.A. (2001) Adenoviral brain-derived neurotrophic factor induces both neostriatal and olfactory neuronal recruitment from endogenous progenitor cells in the adult forebrain. *J. Neurosci.* 21: 6718-6731.
- Bernheimer H., Birkmayer W., Hornykiewicz O., Jellinger K., and Seitelberger F. (1973) Brain dopamine and the syndromes of Parkinson and Huntington. Clinical, morphological and neurochemical correlations. *J. Neurol. Sci.* 20: 415-455.
- Blum D, Torch S, Lambeng N, Nissou M, Benabid AL, Sadoul R, Verna JM (2001) Molecular pathways involved in the neurotoxicity of 6-OHDA, dopamine and MPTP: contribution to the apoptotic theory in Parkinson's disease. *Prog. Neurobiol.* 65:135-172.
- Blum M. (1998) A null mutation in TGF- α leads to a reduction in midbrain dopaminergic neurons in the substantia nigra. *Nat. Neurosci.* 1: 374-377.
- Boewencamp, K.E., Hoffman, A.F., Gerhardt, G.A., Henry, M.A., Biddle, P.T., Hoffer, B.J., and Granholm, A.-C.E. (1995). Glial cell line-derived neurotrophic factor supports survival of injured midbrain dopaminergic neurons. *J. Comp. Neurol.* 355: 479 -489.
- Bonfanti L, Olive S., Poulain D.A. and Theodosis D.T. (1992) Mapping of the distribution of polysialylated neural cell adhesion molecule throughout the central nervous system of the adult rat: an immunohistochemical study. *Neuroscience* 49: 419-436.

- Buckland M.E. and Cunningham A.M. (1999) Alterations in expression of the neurotrophic factors glial cell line-derived neurotrophic factor, ciliary neurotrophic factor and brain-derived neurotrophic factor, in the target-deprived olfactory neuroepithelium. *Neuroscience* 90(1): 333-347.
- Buj-Bello A., Buchman V.L., Horton A., Rosenthal A., Davies A.M. (1995) GDNF is an age-specific survival factor for sensory and autonomic neurons. *Neuron* 15(4): 821-8.
- Breese G.R. and Traylor T.D. (1970) Effect of 6-hydroxydopamine on brain norepinephrine and dopamine: evidence for selective degeneration of catecholamine neurons. *J. Pharmacol. Exp. Ther.* 174(3): 413-420.
- Cameron H.A., Woolley C.S., McEwan B.S., and Gould E. (1993) Differentiation of newly born neurons and glia in the dentate gyrus of the adult rat. *Neuroscience* 56: 337–344.
- Carman L.S., Gage F.H., and Shults C.W. (1991) Partial lesion of the substantia nigra: relation between extent of lesion and rotational behavior. *Brain Res.* 553: 275-83.
- Cass W.A. (1996) GDNF selectively protects dopamine neurons over serotonin neurons against the neurotoxic effects of methamphetamine. *J. Neurosci.* 16: 8132–8139.
- Cass W.A. and Manning M.W. (1999) GDNF protection against 6-OHDA-induced reductions in potassium-evoked overflow of striatal dopamine. *J. Neurosci.* 19: 1416–1423.
- Chazal G., Durbec P., Jankovski A., Rougon G., and Cremer H. (2000). Consequences of neural cell adhesion molecule deficiency on cell migration in the rostral migratory stream of the mouse. *J. Neurosci.* 20: 1446–1457.

- Choi-Lundberg D.L. and Bohn M.C. (1995) Ontogeny and distribution of glial cell line-derived neurotrophic factor (GDNF) mRNA in rat. *Dev. Brain Res.* 85(1):80-88.
- Choi-Lundberg D.L., Lin Q., Chang Y.-N., Chiang Y.L., Hay C.M., Mohajeri H., Davidson B.L. and Bohn M.C. (1997) Dopaminergic neurons protected from degeneration by GDNF gene therapy. *Science* **275**: 838–841.
- Cohen G. and Werner P. (1994) Free radicals, oxidative stress, and neurodegeneration. In *Neurodegenerative Diseases*, D.B. Calne, ed (Philadelphia: W.B. Saunders), pp. 139-161.
- Cohen A.D., Tillerson J.L., Smith A.D., Schallert T., and Zigmond M.J. (2003) Neuroprotective effects of prior limb use in 6-hydroxydopamine-treated rats: possible role of GDNF. *J. Neurochem.* 85: 299-305.
- Collier T.J. and Sortwell C.E. (1999) Therapeutic potential of nerve growth factors in Parkinson's disease. *Drugs Aging* 14: 261-287.
- Conover J.C., Doetsch F., Garcia-Verdugo J.M., Gale N.W., Yancopoulos G.D., and Alvarez-Buylla A. (2000) Disruption of Eph/ephrin signaling affects migration and proliferation in the adult subventricular zone. *Nat. Neurosci.* 3: 1091-1097.
- Cooper O. and Isacson O. (2004) Intrastriatal transforming growth factor α delivery to a model of Parkinson's disease induces proliferation and migration of endogenous adult neural progenitor cells without differentiation into dopaminergic neurons. *J. Neurosci.* 24(41): 8924-8931.
- Craig C.G., Tropepe V., Morshead C.M. Reynolds B.A., Weiss S., and van der Kooy D. (1996) *In vivo* growth factor expansion of endogenous subependymal neural precursor cell populations in the adult mouse brain. *J. Neurosci.* 16: 2649-2658.

- Cremer H., Lange R., Christoph A., Plomann M., Vopper G., Roes J., Brown R., Baldwin S., Kraemer P., Scheff S., et al. (1994) Inactivation of the NCAM gene in mice results in size reduction of the olfactory bulb and deficits in spatial learning. *Nature* 367: 455–459.
- Cremer H., Chazal G., Goridis C., and Represa A. (1997). NCAM is essential for axonal growth and fasciculation in the hippocampus. *Mol. Cell. Neurosci.* 8: 323–335.
- Crespo D., Stanfield B.B., and Cowan W.M. (1986) Evidence that late-generated granule cells do not simply replace earlier formed neurons in the rat dentate gyrus. *Exp. Brain Res.* 62: 541–548.
- Crossin K.L., and Krushel L.A. (2000) Cellular signaling by neural cell adhesion molecules of the immunoglobulin superfamily. *Dev. Dyn.* 218: 260–279.
- Daszuta A., Hery M., Banasr M., Hery F., and Brezun J. (2001) Serotonin mediates estrogen stimulation of adult cell proliferation in the rat dentate gyrus. *Soc. Neurosci. Abstr.* 141: 1.
- Deloulme J.C., Lucas M., Gaber C., Bouillon P., Keller A., Eclancher F., and Sensenbrenner M. (1996) Expression of the neuron-specific enolase gene by rat oligodendroglial cells during their differentiation. *J. Neurochem.* 66(3): 936-45.
- Doetsch F., Caille I., Lim D.A., Garcia-Verdugo J.M., and Alvarez-Buylla A. (1999a) Subventricular zone astrocytes are neuronal stem cells in the adult mammalian brain. *Cell* 97: 703–716.
- Doetsch F., Garcia-Verdugo J.M., and Alvarez-Buylla A. (1999b) Regeneration of a germinal layer in the adult mammalian brain. *Proc. Natl. Acad. Sci. USA.* 96: 11619-11624.

- Doetsch F., Petreanu L., Caille I., Garcia-Verdugo J.M., and Alvarez-Buylla A. (2002) EGF converts transit-amplifying neurogenic precursors in the adult brain into multipotent stem cell. *Neuron* 36: 1021-1034.
- Dunnett S.B., Bjorklund A., and Lindvall O. (2001) Cell therapy in Parkinson's disease - stop or go? *Nat. Rev. Neurosci.* 2(5): 365-9.
- Durbec P., Marcos-Gutierrez C.V., Kilkenny C., Grigoriou M., Wartowaara K., Suvanto P., and Smith D. (1996) GDNF signalling through the Ret receptor tyrosine kinase. *Nature* 381:789-793.
- Eketjall S., Fainzilber M., Murray-Rust J., and F.Ibanez C. (1999) Distinct structural elements in GDNF mediate binding to GFR α 1 and activation of the GFR α 1-c-RET receptor complex. *EMBO J.* 18(21): 5901-5910.
- Fallon J., Reid S., Kinyamu R., Opole I., Opole R., Baratta J., Korc M., Endo T.L., Duong A., Nguyen G., Karkehabadhi M., Twardzik D., Patel S., and Loughlin S. (2000) *In vivo* induction of massive proliferation, directed migration, and differentiation of neural cells in the adult mammalian brain. *Proc. Natl. Acad. Sci. USA.* 97(26): 14686-14691.
- Filippov V., Kronenberg G., Pivneva T., Reuter K., Steiner B., Wang L.-P., Yamaguchi M., Kettenmann H., and Kempermann G. (2003) Subpopulation of nestin-expressing progenitor cells in the adult murine hippocampus shows electrophysiological and morphological characteristics of astrocytes. *Mol. Cell. Neurosci.* 23: 373-382.
- Fleischman R.W., McCracken D., and Forbes W. (1977) Adynamic ileus in the rat induced by chloral hydrate. *Lab Anim. Sci.* 27(2):210-6.

- Fox C.M., Gash D.M., Smoot M.K., Cass W.A. (2001) Neuroprotective effects of GDNF against 6-OHDA in young and aged rats. *Brain Res.* 896:56-63.
- Fricker R.A. et al. (1999) Site-specific migration and neuronal differentiation of human neural progenitor cells after transplantation in the adult rat brain. *J. Neurosci.* 19: 5990-6005.
- Frielingsdorf H., Schwarz K., Brundin P., and Mohapel P. (2004) No evidence for new dopaminergic neurons in the adult mammalian substantia nigra. *Proc. Natl. Acad. Soc. USA.* 101(27): 10177-10182.
- Gage F.H., Coates P.W., Palmer T.D., Kuhn H.G., Fisher L.J., Suhonen J.O., Peterson D.A., Suhr S.T., Ray J. (1995) Survival and differentiation of adult neuronal progenitor cells transplanted to the adult brain. *Proc. Natl. Acad. Sci. USA.* 92: 11879-11882.
- Garcia-Verdugo, J.M., Doetsch, F., Wichterle, H., Lim, D.A., and Alvarez-Buylla, A. (1998). Architecture and cell types of the adult subventricular zone: in search of the stem cells. *J. Neurobiol.* 36(2): 234-48.
- Garcia A.D., Doan N.B., Imura T., Bush T.G., and Sofroniew M.V. (2004) GFAP-expressing progenitors are the principal source of constitutive neurogenesis in adult mouse forebrain. *Nat. Neurosci.* 7(11): 1233-1240.
- Gash, D.M., Zhang, Z., Ovadia, A., Cass, W.A., Yi, A., Simmerman, L., Russell, D., Martin, D., Lapchak, P.A., Collins, F., Hoffer, B.J., and Gerhardt, G. A. (1996) Functional recovery in parkinsonian monkeys treated with GDNF. *Nature* 380: 252 -255.

- Gerlai R., McNamara A., Choi-Lundberg D.L., Armanini M., Ross J., Powell-Braxton L., and Phillips H.S. (2001) Impaired water maze learning performance without altered dopaminergic function in mice heterozygous for the GDNF mutation. *Europ. J. Neurosci.* 14: 1153-1163.
- Gill S.S., Patel N.K., Hotton G.R., O'Sullivan K., McCarter R., Bunnage M., Brooks D.J., Svendsen C.N., and Heywood P. (2003) Direct brain infusion of glial cell line-derived neurotrophic factor in Parkinson disease. *Nat. Med.* 9(5): 589-595.
- Goldman S.A. (1998). Adult neurogenesis: from canaries to the clinic. *J. Neurobiol.* 36(2): 267-86.
- Gotz R. and Scharf M. (1994). The conservation of neurotrophic factors during vertebrate evolution. *Comp. Biochem. Physiol. Pharmacol. Toxicol. Endocrinol.* 108(1): 1-10.
- Gould E., Cameron H.A., Daniels, D.C., Woolley C.S., and McEwen B.S. (1992) Adrenal hormones suppress cell division in the adult rat dentate gyrus. *J. Neurosci.* 12:3642–3650.
- Gould E., Cameron H.A., and McEwen B.S. (1994) Blockade of NMDA receptors increases cell death and birth in the developing rat dentate gyrus. *J. Comp. Neurol.* 340:551–565.
- Gould E., and Tanapat P. (1997) Lesion-induced proliferation of neuronal progenitor cells in the dentate gyrus of the adult rat. *Neurosci.* 80: 427-436.

- Granholt A.C., Reyland M., Albeck D., Sanders L., Gerhardt G., Hoernig G., Shen L., Westphal H., and Hoffer B. (2000) Glial cell line-derived neurotrophic factor is essential for postnatal survival of midbrain dopamine neurons. *J. Neurosci.* 20(9): 3182-3190.
- Grondin R., Zhang Z., Yi A., Cass W.A., Maswood N., Andersen A.H., Elsberry D.D., Klein M.C., Gerhardt G.A., Gash D.M. (2002) Chronic, controlled GDNF infusion promotes structural and functional recovery in advanced parkinsonian monkeys. *Brain.* 125(Pt 10): 2191-2201.
- Grondin R., Cass W.A., Zhang Z., Stanford J.A., Gash D.M., and Gerhardt G.A. (2003) Glial cell line-derived neurotrophic factor increases stimulus-evoked dopamine release and motor speed in aged rhesus monkeys. *J. Neurosci.* 23(5): 1974-1980.
- Hall M.E., Hoffer B.J., and Gerhardt G.A. (1989) Rapid and sensitive determination of catecholamines in small tissue samples by high performance liquid chromatography coupled with dual-electrode coulometric electrochemical detection. *LC/GC* 7(3): 258-265.
- Harding A.J., Halliday G.M., Cullen K. (1994) Practical considerations for the use of the optical dissector in estimating neuronal number. *J. Neurosci. Methods* 51: 83–89.
- Hellmich H.L., Kos L., Cho E.S., Mahon KA, Zimmer A. (1996) Embryonic expression of glial cell-line derived neurotrophic factor (GDNF) suggests multiple developmental roles in neural differentiation and epithelial-mesenchymal interactions. *Mech. Dev.* 54(1):95-105.

- Hely M.A., Morris J.G., Traficante R., Reid W.G., O'Sullivan D.J., Williamson P.M. (1999) The sydney multicentre study of Parkinson's disease: progression and mortality at 10 years. *J. Neurol. Neurosurg. Psychiatry* 67: 300-307.
- Henderson C.E., Phillips H.S., Pollock R.A., Davies A.M., Lemeulle C., Armanini M., Simmons L., Moffet B., Vandlen R.A., and Simpson L.C. (1994) GDNF: a potent survival factor for motoneurons present in peripheral nerve and muscle. *Science* 266(5187):1062-1064.
- Henry M.A., Westrum L.E., and Johnson L.R. (1985) Ultrastructure of transganglionic HRP transport in cat trigeminal system. *Brain Res.* 334(2): 255-266.
- Hoehn M.M. and Yahr M.D. (1998) Parkinsonism: onset, progression, and mortality. *Neurology* 50(2): 318-333.
- Hoffer B.J., Hoffman A., Bowenkamp K., Huettl P., Hudson J., Martin D., Lin L.-F.H. and Gerhardt G.A. (1994) Glial cell line-derived neurotrophic factor reverses toxin-induced injury to midbrain dopaminergic neurons *in vivo*. *Neurosci. Lett.* 182: 107–111.
- Hoglinger G.U., Rizk P., Muriel M.P., Duyckaerts C., Oertel W.H., Caille I., and Hirsch E.C. (2004) Dopamine depletion impairs precursor cell proliferation in Parkinson disease. *Nat. Neurosci.* 7(7):726-735.
- Horner P.J. and Gage F.H. (2000) Regenerating the damaged central nervous system. *Nature* 407: 963-970.
- Hudson J.L., Levin D.R., and Hoffer B.J. (1993). A 16-channel automated rotometer system for reliable measurement of turning behavior in 6-hydroxydopamine lesioned and transplanted rats. *Cell Transplant.* 2(6): 507-514.

- Hudson, J., Granholm, A.-C. E., Gerhardt, G. A., Henry, M. A., Hoffman, A. F., Biddle, P. T., Leela, N. S., Mackerlova, L., Lile, J. D., Collins, F., and Hoffer, B. J. (1995). Glial cell line-derived neurotrophic factor augments midbrain dopaminergic circuits *in vivo*. *Brain Res. Bull.* 36: 425 - 432.
- Ishikawa K., Ott T., and McGaugh J.L. (1982) Evidence for dopamine as a transmitter in dorsal hippocampus. *Brain Res.* 232: 222-226.
- Iwai M., Sato K., Kamada H., Omori N., Nagano I., Shoji M., and Abe K. (2003) Temporal profile of stem cell division, migration, and differentiation from subventricular zone to olfactory bulb after transient forebrain ischemia in gerbils. *J. Cereb. Blood Flow Metab.* 23: 331-341.
- Jellinger K.A. (2001) The pathology of Parkinson's disease. *Adv. Neurol.* 86: 55-72.
- Jeon B.S., Jackson-Lewis V., and Burke R.E. (1995) 6-hydroxydopamine lesion of the rat substantia nigra: Time course and morphology of cell death. *Neurodegeneration* 4: 131-137.
- Jing S., Wen D., Yu Y., Holst P.L., Luo Y., Fang M., and Tamir R. (1996) GDNF-induced activation of the ret protein tyrosine kinase is mediated by GDNFR-alpha, a novel receptor for GDNF. *Cell* 85:1113-1124.
- Kearns, C.M. and Gash, D.M. (1995). GDNF protects nigral dopamine neurons against 6-hydroxydopamine *in vivo*. *Brain Res.* 672: 104 -111.
- Kearns, C.M., Cass, W.A., Smoot, K., Kryscio, R., and Gash, D.M. (1997). GDNF protection against 6-OHDA: Time dependence and requirement for protein synthesis. *J. Neurosci.* 17(18): 7111-7118.

- Kempermann G., Kuhn H.G., and Gage F.H. (1997) More hippocampal neurons in adult mice living in an enriched environment. *Nature* 386: 493-495.
- Kempermann G., Kuhn H.G., and Gage F.H. (1998) Experience-induced neurogenesis in the senescent dentate gyrus. *J. Neurosci.* 18: 3206-3212.
- Kirik D., Rosenblad C., and Bjorklund A. (1998) Characterization of behavioral and neurodegenerative changes following partial lesions of the nigrostriatal dopamine system induced by intrastriatal 6-hydroxydopamine in the rat. *Exp. Neurol.* 152(2): 259-77.
- Kish S.J., Shannak K., and Hornykiewicz O. (1988) Uneven pattern of dopamine loss in the striatum of patients with idiopathic Parkinson's disease. Pathophysiologic and clinical implications. *N. Engl. J. Med.* 318: 876-880.
- Kokaia Z., Airaksinen M.S., Nanoshvili A., Larsson E., Kujamliki E., Lindvall O., and Saarma M. (1999) GDNF family ligands and receptor are differentially regulated after brain insults in the rat. *Eur. J. Neurosci.* 11: 1202-1216.
- Kornack D.R. and Rakic P. (2001) Cell proliferation without neurogenesis in adult primate neocortex. *Science* 294: 2127-2130.
- Kornblum H.I., Hussain R.J., Bronstein J.M., Gall C.M., Lee D.C., and Seroogy K.B. (1997) Prenatal ontogeny of the epidermal growth factor receptor and its ligand, transforming growth factor alpha, in the rat brain. *J. Comp. Neurol.* 380: 243-261.

- Kordower J.H., Emborg M.E., Bloch J., Ma S.Y., Chu Y., Leventhal L., McBride J., Chen E.Y., Palfi S., Roitberg B.Z., Brown W.D., Holden J.E., Pyzalski R., Taylor M.D., Carvey P., Ling Z., Trono D., Hantraye P., Deglon N., and Aebischer P. (2000) Neurodegeneration prevented by lentiviral vector delivery of GDNF in primate models of Parkinson's disease. *Science* 290(5492): 767-773.
- Kotzbauer P.T., Lampe P.A., Heuckeroth R.O., Golden J.P., Creedon D.J., Johnson E.M. Jr, and Milbrandt J. (1996) Neurturin, a relative of glial-cell-line-derived neurotrophic factor. *Nature* 384(6608): 467-70.
- Krack P., Pollak P., Limousin P., Benazzouz A., Deuschl G., and Benabid A.L. (1999) From off-period dystonia to peak-dose chorea. The clinical spectrum of varying subthalamic nucleus activity. *Brain*. 122 (Pt 6): 1133-1146.
- Kuhn H.G., Dickinson-Anson H., and Gage F.H. (1996) Neurogenesis in the dentate gyrus of the adult rat: age-related decrease of neuronal progenitor proliferation. *J. Neurosci.* 16: 2027-2033.
- Kuhn H.G., Winkler J., Kempermann G., Thal L.J., and Gage F.H. (1997). Epidermal growth factor and fibroblast growth factor-2 have different effects on neural progenitors in the adult rat brain. *J. Neurosci.* 17: 5820-5829.
- Lang A.E. and Lozano AM (1998a) Parkinson's disease. First of two parts. *N. Engl. J. Med.* 339: 1044-1053.
- Lang A.E. and Lozano AM (1998b) Parkinson's disease. Second of two parts. *N. Engl. J. Med.* 339: 1130-1143.

- Lapchak P.A., Miller P.J., and Jiao S. (1997) Glial cell line-derived neurotrophic factor induces the dopaminergic and cholinergic phenotype and increases locomotor activity in aged Fisher 344 rats. *Neuroscience* 77: 745–752.
- Laywell E.D., Rakic P., Kukekov V.G., Holland E.C., and Steindler D.A. (2000) Identification of a multipotent astrocytic stem cell in the immature and adult mouse brain. *Proc. Natl. Acad. Sci. USA*. 97: 13883-13888.
- Leavitt B.R., Hearn-Grant C.S., Macklis J.D. (1999) Mature astrocytes transform into transitional radial glia within adult mouse neocortex that supports directed migration of transplanted immature neurons. *Exp. Neurol.* 157(1): 43-57.
- Lendahl U., Zimmerman L.B., McKay R.D.G. (1990) CNS stem cells express a new class of intermediate filament protein. *Cell* 60: 585-595.
- Leonardi E.T. and Mytilineou C. (1998) Cell culture models of neuronal degeneration and neuroprotection. Implications for Parkinson's disease. In *Molecular and cellular mechanisms of neuronal plasticity : basic and clinical implications*, Y.H. Ehrlich, ed (New York : Kluwer Academic/Plenum Publishers), pp. 203-222.
- Li Z. Kato T., Kawagishi K., Fukushima N., Yokouchi K., and Moriizumi T. (2002) Cell dynamics of calretinin-immunoreactive neurons in the rostral migratory stream after ibotenate-induced lesions in the forebrain. *Neurosci. Res.* 42: 123-132.
- Lie D.C., Dzieczapolski G., Willhoite A.R., Kaspar B.K., Shults C.W. and Gage F.H. (2002) The adult substantia nigra contains progenitor cells with neurogenic potential. *J. Neurosci.* 22(15): 6639-6649.

- Lin L.F., Doherty D., Lile J., Bektess S., and Collins F. (1993) GDNF: a glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. *Science* 260: 1130 -1132.
- Lin L.F. (1996) Glial cell line-derived neurotrophic factor (GDNF): a comprehensive review. *Neural Notes* 7: 3-7.
- Lindsay R.M., Altar C.A., Cedarbaum J.M., Hyman C., Wiegand S.J. (1993) The therapeutic potential of neurotrophic factors in the treatment of Parkinson's disease. *Exp. Neurol.* 124:103-118.
- Lindvall O., Kokaia Z., Bengzon J., Elmer E., and Kokaia M. (1994) Neurotrophins and brain insults. *Trends. Neurosci.* 17(11): 490-496.
- Lindvall O. and McKay R. (2003) Brain repair by cell replacement and regeneration *Proc. Natl. Acad. Sci. USA.* 100: 7430-7431.
- Lindvall O., Kokaia Z., and Martinez-Serrano A. (2004) Stem cell therapy for human neurodegenerative disorders-how to make it work. *Nat. Med.* Suppl: S42-50.
- Liu J., Solway K., Messing R.O., and Sharp F.R. (1998) Increased neurogenesis in the dentate gyrus after transient global ischemia in gerbils. *J. Neurosci.* 18: 7768-7778.
- Lois C. and Alvarez-Buylla A. (1994) Long-distance neuronal migration in the adult mammalian brain. *Science* 264: 1145–1148.
- Luskin M.B. (1993) Restricted proliferation and migration of postnatally generated neurons derived from the forebrain subventricular zone. *Neuron* 11(1): 173–189.
- Magavi S.S., Leavitt B.R., and Macklis J.D. (2000) Induction of neurogenesis in the neocortex of adult mice. *Nature* 405(6789): 951-955.

- Mao L, Lau Y.S., Petroske E., and Wang J.Q. (2001) Profound astrogenesis in the striatum of adult mice following nigrostriatal dopaminergic lesion by repeated MPTP administration. *Dev. Brain Res.* 131: 57-65.
- McKay R. (1997) Stem cells in the central nervous system. *Science* 276: 66-71.
- Mendez, J.S. and Finn, B.W. (1975). Use of 6-hydroxydopamine to create lesions in catecholamine neurons in rats. *J. Neurosurg.* 42(2): 166-73.
- Milbrandt J., de Sauvage F.J., Fahrner T.J., Baloh R.H., Leitner M.L., Tansey M.G., Lampe P.A., Heuckeroth R.O., Kotzbauer P.T., Simburger K.S., Golden J.P., Davies J.A., Vejsada R., Kato A.C., Hynes M., Sherman D., Nishimura M., Wang L.C., Vandlen R., Moffat B., Klein R.D., Poulsen K., Gray C., Garces A., and Johnson E.M. Jr, et al. (1998) Persephin, a novel neurotrophic factor related to GDNF and neurturin. *Neuron* 20(2): 245-53.
- Moller J.C., Sautter J., and Kupsch A. (1996) Potential of neurotrophic factors in therapy of Parkinson's disease. *J. Neural. Transm.-Suppl.* 48:103-112.
- Morshead C.M. and van der Kooy D (1990) Separate blood and brain origins of proliferating cells during gliosis in adult brains. *Brain Res.* 535: 237-244.
- Morshead C.M. and van der Kooy D. (1992) Postmitotic death is the fate of constitutively proliferating cells in the subependymal layer of the adult mouse brain. *J. Neurosci.* 12: 249-256.
- Mount H.T., Dean D.O., Alberch J., Dreyfus C.F., Black I.B. (1995) Glial cell line-derived neurotrophic factor promotes the survival and morphologic differentiation of Purkinje cells. *Proc. Natl. Acad. Sci. USA.* 92(20):9092-9096.

- Mullen R.J., Buck C.R., and Smith A.M. (1992). NeuN, a neuronal specific nuclear protein in vertebrates. *Development* 116(1): 201-11.
- Murphy M., Drago J., and Bartlett P.F. (1990) Fibroblast growth factor stimulates the proliferation and differentiation of neural precursor cells *in vitro*. *J. Neurosci. Res.* 25, 463–475.
- Nakao N., Nakai K., and Itakura T. (2000) Fetal striatal transplants reinstate the electrophysiological response of pallidal neurons to systemic apomorphine challenge in rats with excitotoxic striatal lesions. *Eur. J. Neurosci.* 12(9): 3426-3432.
- Nakatomi H., Kuriu T., Okabe S., Yamamoto S., Hatano O., Kawahara N., Tamura A., Kirino T., and Nakafuku M. (2002) Regeneration of hippocampal pyramidal neurons after ischemic brain injury by recruitment of endogenous neural progenitors. *Cell* 110: 429–41.
- Nishiyama A., Chang A. and Trapp B.D. (1999) NG2+ glial cells: a novel glial cell population in the adult brain. *J. Neuropathol. Exp. Neurol.* **58**, 1113–1124.
- Nowakowski R.S. and Hayes N.L. (2000) New neurons: extraordinary evidence or extraordinary conclusion? *Science* 288(5467): 771a.
- Nowakowski R.S. and Hayes N.L. (2002) Stem Cells: The Promises and Pitfalls. *Neuropsychopharmacol.* 25: 799-804.
- Okano H.J., Pfaff D.W., and Gibbs R.B. (1996) Expression of EGFR-, p75NGFR-, and PSTAIR (cdc2)-like immunoreactivity by proliferating cells in the adult rat hippocampal formation and forebrain. *Dev. Neurosci.* 18(3): 199-209.

- Olson L. and Malmfors T. (1970) Growth characteristics of adrenergic nerves in the adult rat. Fluorescence histochemical and 3H-noradrenaline uptake studies using tissue transplantations to the anterior chamber of the eye. *Acta Physiol. Scand. Suppl.* 348: 1-112.
- Olucha F., Martinez-Garcia F., and Lopez-Garcia C. (1985) A new stabilizing agent for the tetramethyl benzidine (TMB) reaction product in the histochemical detection of horseradish peroxidase (HRP). *J. Neurosci Methods.* 13(2):131-138.
- Oppenheim R.W., Houenou L.J., Johnson J.E., Lin L.F., Li L., Lo A.C., Newsome A.L., Prevette D.M., and Wang S. (1995) Developing motor neurons rescued from programmed and axotomy-induced cell death by GDNF. *Nature* 373, 344-346.
- Ormerod B.K. and Galea L.A. (2001) Reproductive status influences cell proliferation and cell survival in the dentate gyrus of adult female meadow voles: a possible regulatory role for estradiol. *Neuroscience* 102: 369–379.
- Palmer T.D., Ray J., and Gage F.H. (1995) FGF-2-responsive neuronal progenitors reside in proliferative and quiescent regions of the adult rodent brain. *Mol. Cell. Neurosci.* 6: 474-486.
- Palmer T.D., Takahashi J., and Gage F.H. (1997) The adult rat hippocampus contains primordial neural stem cells. *Mol. Cell. Neurosci.* 8: 389-404.
- Palmer T.D., Willhoite A.R., Gage F.H. (2000) Vascular niche for adult hippocampal neurogenesis. *J. Comp. Neurol.* 425: 479-494.
- Paratcha G., Ledda F., and Ibanez C.F. (2003) The neural cell adhesion molecule NCAM is an alternative signaling receptor for GDNF family ligands. *Cell.* 113:867-79.

- Parent J.M., Yu T.W., Leibowitz R.T., Geschwin, D.H., Sloviter R.S., and Lowenstein D.H. (1997) Dentate granule cell neurogenesis is increased by seizures and contributes to aberrant network reorganization in the adult rat hippocampus. *J. Neurosci.* 17: 3727-3738.
- Parent J.M., Valentin V.V., and Lowenstein D.H. (2002) Prolonged seizures increase proliferating neuroblasts in the adult rat subventricular zone-olfactory bulb pathway. *J. Neurosci.* 22: 3174-3188.
- Pelleymounter M.A., Cullen M.J., Baker M.B. and Healy D. (1999) Glial cell line-derived neurotrophic factor (GDNF) improves spatial learning in aged Fisher 344 rats. *Psychobiology.* 27: 397-401.
- Pencea V., Bingaman K.D., Wiegand S.J., Luskin M.B. (2001) Infusion of brain-derived neurotrophic factor into the lateral ventricle of the adult rat leads to new neurons in the parenchyma of the striatum, septum, thalamus, and hypothalamus. *J. Neurosci.* 21: 6706–17.
- Quintero E.M., Willis L.M., Zaman V., Lee J., Boger H.A., Tomac A., Hoffer B.J., Stromberg I., and Granholm A.C. (2004) Glial cell line-derived neurotrophic factor is essential for neuronal survival in the locus coeruleus-hippocampal noradrenergic pathway. *Neuroscience* 124(1): 137-46.
- Rakic P. Neurogenesis in adult primate neocortex: an evaluation of the evidence. (2002) *Nat. Rev. Neurosci.* 3: 65-71.
- Rascol O., Payoux P., Ory F., Ferreira J.J., Brefel-Courbon C., and Montastruc JL (2003) Limitations of current Parkinson's disease therapy. *Ann. Neurol.* 53 (Suppl 3): S3-12; discussion S12-15.

- Reeben M., Laurikainen A., hiltunen J.O., Castren E., and Saarma M. (1998) The messenger RNAs for both glial cell line-derived neurotrophic factor receptors, c-ret and GDNFRalpha, are induced in the rat brain in response to Kainate-induced excitation. *Neuroscience* 83: 151-159.
- Reynolds B.A., Tetzlaff W. and Weiss S. (1992) A multipotent EGF-responsive striatal embryonic progenitor cell produces neurons and astrocytes. *J. Neurosci.* 12, 4565–4574.
- Reynolds B.A. and Weiss S. (1992) Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science* 255(5052): 1707-1710.
- Richards L.J., Kilpatrick T.J., and Bartlett P.F. (1992) De novo generation of neuronal cells from the adult mouse brain. *Proc. Natl. Acad. Sci. USA.* 89: 8591-8595.
- Richardson P.M., McGuinness U.M., and Aguayo A.J. (1980) Axons from CNS neurons regenerate into PNS grafts. *Nature* 284: 264-265.
- Rosenthal A. (1999) The GDNF protein family: gene ablation studies reveal what they really do and how. *Neuron* 22(2): 201-203.
- Rosser A.E., Tyers P., Borg ter M., Dunnett S.B., and Svendsen C.N. (1997) Co-expression of MAP-2 and GFAP in cells developing from rat EGF responsive precursor cells. *Dev. Brain Res.* 98(2):291-295.
- Rye D.B., Saper C.B., and Wainer B.H. (1984) Stabilization of the tetramethylbenzidine (TMB) reaction product: application for retrograde and anterograde tracing, and combination with immunohistochemistry. *J. Histochem. Cytochem.* 32(11): 1145-1153.

- Sarabi A., Hoffer B.J., Olson L., and Morales M. (2000) GFR alpha-1 is expressed in parvalbumin GABAergic neurons in the hippocampus. *Brain Res.* 877(2): 262-270.
- Sarabi A., Hoffer B.J., Olson L., and Morales M. (2001) GFRalpha-1 mRNA in dopaminergic and nondopaminergic neurons in the substantia nigra and ventral tegmental area. *J. Comp. Neurol.* 441(2):106-117.
- Sarabi A., Hoffer B.J., Olson L., and Morales M. (2003) Glial cell line neurotrophic factor-family receptor alpha-1 is present in central neurons with distinct phenotypes. *Neuroscience* 116(1): 261-273.
- Sauer H., Rosenblad C. and Bjorklund A. (1995) Glial cell line-derived neurotrophic factor but not transforming growth factor β 3 prevents delayed degeneration of nigral dopaminergic neurons following striatal 6-hydroxydopamine lesions. *Proc. Natl. Acad. Sci. USA.* 92: 8935–8939.
- Segal R.A. and Greenberg M.E. (1996) Intracellular signaling pathways activated by neurotrophic factors. *Annu. Rev. Neurosci.* 19: 463-489.
- Sensenbrenner M., Lucas M., and Deloulme J.C. (1997). Expression of two neuronal markers, growth-associated protein 43 and neuron-specific enolase, in rat glial cells. *J. Mol. Med.* 75(9): 653-63.
- Seri B., Garcia-Verdugo J.M., McEwen B.S., and Alvarez-Buylla A. (2001) Astrocytes give rise to new neurons in the adult mammalian hippocampus. *J. Neurosci.* 21(18): 7153-7160.
- Seroogy K.B., Numan S., Gall C.M., Lee D.C., and Kornblum H.I. (1994) Expression of EGF receptor mRNA in rat nigrostriatal system. *NeuroReport* 6(1):105-8.

- Seroogy K.B., Gall C.M., Lee D.C., Kornblum H.I. (1995) Proliferative zones of postnatal rat brain express epidermal growth factor receptor mRNA. *Brain Res.* 670(1): 157-164.
- Shors T.J., Miesegaes G., Beylin A., Zhao M., Rydel T., and Gould E. (2001) Neurogenesis in the adult is involved in the formation of trace memories. *Nature* 410: 372-376.
- Siderowf A. and Stern M. (2003) Update on Parkinson disease. *Ann. Intern. Med.* 138: 651-658.
- Silverman J., Muir W.W. 3rd. (1993) A review of laboratory animal anesthesia with chloral hydrate and chloralose. *Lab. Anim. Sci.* 43(3): 210-216.
- Slevin J., Young A.B., Gash D.M., and Gerhardt G. (2004) Unilateral intraputamenal administration of recombinant-methionyl human glial cell line-derived neurotrophic factor to treat idiopathic Parkinson disease. Oral presentation 765. AANS annual meeting.
- Steece-Collier K., Maries E., and Kordower J.H. (2002) Etiology of Parkinson's disease: Genetics and environment revisited. *Proc. Natl. Acad. Sci. USA.* 99: 13972-13974.
- Suvanto P., Hiltunen J.O., Arumae U., Moshnyakov M., Sariola H., Sainio K., and Saarma M. (1996) Localization of glial cell line-derived neurotrophic factor (GDNF) mRNA in embryonic rat by in situ hybridization. *Eur. J. Neurosci.* 8(4): 816-822.

- Tanapat P., Hastings N.B., Reeves A.J., and Gould E. (1999) Estrogen stimulates a transient increase in the number of new neurons in the dentate gyrus of the adult female rat. *J. Neurosci.* 19(14):5792-5801.
- Tao Y., Black I.B., and DiCicco-Bloom E. (1996) Neurogenesis in neonatal rat brain is regulated by peripheral injection of basic fibroblast growth factor (bFGF). *J. Comp. Neurol.* 376: 653-663.
- Tomac, A., Lindqvist, E., Lin, L.-F., Ögren, S., Young, D., Hoffer, B., and Olson, L. (1995) Protection and repair of the nigrostriatal dopaminergic system by GDNF *in vivo*. *Nature* 373: 335 -339.
- Tomasiewicz H., Ono K., Yee D., Thompson C., Goridis C., Rutishauser U., and Magnuson T. (1993) Genetic deletion of a neural cell adhesion molecule variant (NCAM-180) produces distinct defects in the central nervous system. *Neuron* 11: 1163–1174.
- Treanor J.J., Goodman L., de S.F., Stone D.M., Poulsen K.T., Beck C.D., Gray C. (1996) Characterization of a multicomponent receptor for GDNF. *Nature* 382:80–83.
- Tropepe V., Craig C.G., Morshead C.M., and van der Kooy D. (1997) Transforming growth factor- α null and senescent mice show decreased neural progenitor cell proliferation in the forebrain subependyma. *J. Neurosci.* 17: 7850-7859.
- Trupp M., Ryden M., Jornvall H., Funakoshi H., Timmusk T., Arenas E., Ibanez C.F. (1995) Peripheral expression and biological activities of GDNF, a new neurotrophic factor for avian and mammalian peripheral neurons. *J. Cell. Biol.* 130(1): 137-148.

- Trupp M., Arenas E., Fainzilber M., Nilsson A.S., Sieber B.A., Grigoriou M., Kilkenny C. (1996) Functional receptor for GDNF encoded by the c-ret proto-oncogene. *Nature* 381:785–788.
- Trupp M, Belluardo N, Funakoshi H and Ibanez CF. (1997) Complementary and overlapping expression of glial cell line-derived neurotrophic factor (GDNF), c-ret proto-oncogene, and GDNF receptor-alpha indicates multiple mechanisms of trophic actions in the adult rat CNS. *J. Neurosci.* 17:3554-3567.
- Trupp M., Raynoschek C., Belluardo N. and Ibanez C.F. (1998) Multiple GPI-anchored receptors control GDNF-dependent and independent activation of the c-Ret receptor tyrosine kinase. *Mol. Cell. Neurosci.* 11:47-63.
- Ungerstedt, U. (1968). 6-hydroxy-dopamine induced degeneration of central monoamine neurons. *Eur. J. Pharmacol.* 5: 107-10.
- Ungerstedt, U. (1971). Postsynaptic supersensitivity after 6-hydroxydopamine induced degeneration of nigro-striatal dopamine system. *Acta Physiol. Scand. Suppl.* 367: 69-93.
- Uretsky, N.J. and Iversen, L.L. (1970). Effects of 6-hydroxydopamine on catecholamine containing neurones in the rat brain. *J. Neurochem.* 17(2): 269-78.
- van Praag H., Schinder A.F., Christie B.R., Toni N., Palmer T.D., and Gage F.H. (2002) Functional neurogenesis in the adult hippocampus. *Nature* 415: 1030-1034.
- Vescovi A.L., Reynolds B.A., Fraser D.D., and Weiss S. (1993) bFGF regulates the proliferative fate of unipotent (neuronal) and bipotent (neuronal/astroglial) EGF-generated CNS progenitor cells. *Neuron* 11, 951–966.

- Wang Y., Sheen V.L., Macklis J.D. (1998) Cortical interneurons upregulate neurotrophins *in vivo* in response to targeted apoptotic degeneration of neighboring pyramidal neurons. *Exp. Neurol.* 154(2): 389-402.
- Wartiovaara K. "GDNF and p75 neurotrophin receptor in development and disease." (Ph.D. dissertation, University of Helsinki, 1998). Online. Available: <http://ethesis.helsinki.fi/julkaisut/laa/biola/vk/wartiovaara/gdnfandp.pdf>. Retrieved: 23 Jan. 2005.
- Weiss S., Reynolds B.A., Vescovi A.L., Morshead C., Craig C.G. and van der Kooy D. (1997) Is there a neural stem cell in the mammalian forebrain? *TINS* 19(9):387-393.
- West M.J., Slomianka L., Gundersen H.J.G. (1991) Unbiased stereological estimation of the total number of neurons in the subdivisions of the rat hippocampus using the optical fractionator. *Anat. Rec.* 231: 482–497.
- West M.J. (1993) New stereological methods in counting neurons. *Neurobiol. Aging* 14: 275–285.
- Yan Q., Matheson C., and Lopez O.T. (1995) *In vivo* neurotrophic effects of GDNF on neonatal and adult facial motor neurons. *Nature* 373, 341-344.
- Young D., Lawlor P.A., Leone P., Dragunow M., and During M.J. (1999) Environmental enrichment inhibits spontaneous apoptosis, prevent seizures and is neuroprotective. *Nat. Med.* 5: 448-453.

- Zhao M., Momma S., Delfani K., Carlen M., Cassidy R.M., Johansson C.B., Brismar H., Shupliakov O., Frisen J., Janson A.M. (2003) Evidence for neurogenesis in the adult mammalian substantia nigra. *Proc. Natl. Acad. Sci. USA*. 100(13): 7925-7930.
- Zigova T., Pencea V., Wiegand S.J., and Luskin M.B. (1998) Intraventricular administration of BDNF increases the number of newly generated neurons in the adult olfactory bulb. *Mol. Cell. Neurosci*. 11: 234-245.

VITA

YAN CHEN

EDUCATION AND PROFESSIONAL TRAINING

- Sep. 1999 – Present Dept. of Anatomy & Neurobiology, University of Kentucky
47 Davis Mills Bldg, 800 Rose Street, Lexington, KY 40536
Ph.D. Candidate in Neuroscience
Cumulative GPA: **3.86**
- Sep. 1996 – July 1999 Department of Physiology, Peking Union Medical College
5 Dong Dan San Tiao, Beijing 100005, China
Master of Science in Neurophysiology
- Sep. 1992 – July 1996 Department of Biology, Beijing Normal University
19 Xijiekouwei Street, Beijing 100857, China
Bachelor of Science in Biology

RESEARCH EXPERIENCE

- Sep. 1999 – Present Dept. of Anatomy & Neurobiology, University of Kentucky
47 Davis Mills Bldg, 800 Rose Street, Lexington, KY 40536
Research Assistant
Research focuses on neurogenesis in Basal Ganglia in
Parkinsonian animal model (6-OHDA lesioned rat model)
and aging animal model.
- Sep. 1996 – July 1999 Department of Physiology, Peking Union Medical College
5 Dong Dan San Tiao, Beijing 100005, China
Master of Science in Neurophysiology
Research focuses on the effect of oxidative stress on the
etiology of Alzheimer's Disease animal model.

PUBLICATIONS

1. **Y. Chen**, Y. Ai, Q. Cai, M.G. Engle, M. Jennes, D.M. Gash. Effects of GDNF continuous infusion on normal rats in the striatum and the substantia nigra. (In Preparation)
2. J.A. Gurwell, A. Nath, Q. Sun, J. Zhang, K.M. Martin, **Y. Chen** and K.F. Hauser. Synergistic neurotoxicity of opioids and human immunodeficiency virus-1 tat protein in striatal neurons in vitro. *Neuroscience*, 2001, 102(3): 555-63.
3. Shiyi Zhang, **Yan Chen**, Pingping Zuo, Bo Yuan, Dan Liu, Yanling Jing and Yan Liu. Therapeutic effect of 764-3 on rat with Alzheimer's like disease. *Basic Medical Sciences and Clinics*, 2001, 21(1): 69-72.
4. **Yan Chen**. Autoimmune Disease and Oral Tolerance (Review). *Natural Journal*, 1999, 21 (3): 135-8.

PRESENTATIONS AND PUBLIC APPEARANCES

1. **Y. Chen**, Q. Cai, M.G. Engle, M. Jennes, Y. Ai, D.M. Gash. BrdU infusion in normal rat labeled progenitor cells in the striatum and the substantia nigra: an electron microscopic study. Program No. 381.4. *2004 Abstract Viewer/Itinerary Planner*. Washington, DC: Society for Neuroscience
2. **Y. Chen**, Y. Ai, D.M. Gash. GDNF infusion enhances adult stem cell proliferation, but not neurogenesis in the 6 - OHDA lesioned rat substantia nigra. Program No. 672.14. *2003 Abstract Viewer/Itinerary Planner*. Washington, DC: Society for Neuroscience
3. **Y. Chen**, Y. Ai, D.M. Gash. GDNF INFUSION ENHANCES ADULT STEM CELL PROLIFERATION IN NORMAL RAT BRAIN. Program No. 329.9. *2002 Abstract Viewer/Itinerary Planner*. Washington, DC: Society for Neuroscience, 2002. CD-ROM.
4. **Yan Chen**, Pingping Zuo, Shiyi Zhang, Bo Yuan and Dan Liu. The treatment of a Chinese medicine, 764-3, on a rat model of Alzheimer's disease. Abstracts of '99 International Symposium on Aging and Anti-aging Science & Technology, September, 1999, Beijing, China, P52.
5. **Yan Chen**, Yan Liu and Hongwei Ren. A rat model induced by β -AP (25-35) could be useful for the study of AD. Abstracts of '99 International Symposium on Aging and Anti-Aging Science & Technology, September, 1999, Beijing, China, P41.
6. **Renren Fei**, Yan Chen, Hui Chen and Shepu Xue. The Chromatinic Reconstruction Study of Spermatid in vitro. Abstracts of National Symposium on Reproductive Biology, 1996, Suzhou, China, P28.

7. The Role of GDNF on Adult Neurogenesis in Normal and 6-OHDA Lesioned Rats. Apr. 21st, 2003. Department of Anatomy & Neurobiology Seminar Series.
8. GDNF and stem cell proliferation. June 10th, 2002. Department of Anatomy & Neurobiology Seminar Series.
9. The Role of GDNF on Adult Neurogenesis in Normal and 6-OHDA Lesioned Rats. Sep. 27th, 2001. Qualifying Proposal.

PROFESSIONAL AFFILIATIONS

Society for Neuroscience, 2001-Present
The American Physiological Society, 2003-Present
International Society for Stem Cell Research, 2004-Present

Yan Chen

March 7, 2005